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P. [US/US]; 2244 El Amigo, Del Mar, CA 92014 (US).
SHOPES, Robert, J. [US/US]; 1639 28th Street, San Diego, CA 92102 (US).

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(74) Agents: **GUISE, Jeffrey, W.** et al.; Brobeck, Phleger & Harrison, LLP, 12390 El Camino Real, San Diego, CA 92130 (US).

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(71) Applicant (*for all designated States except US*):
FAVRILLE, INC. [US/US]; Suite 100, 10835 Altman Row, San Diego, CA 92121 (US).

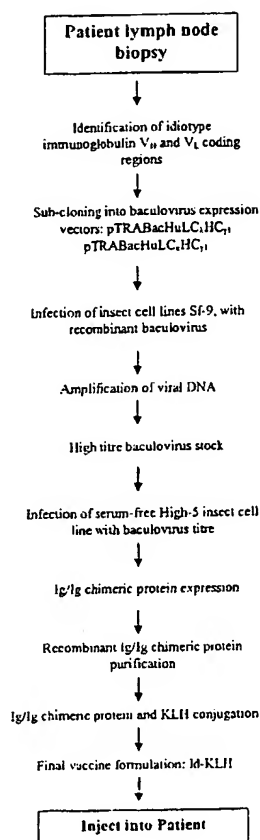
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(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **GOLD, Daniel,**

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(54) Title: METHOD AND COMPOSITION FOR ALTERING A B CELL MEDIATED PATHOLOGY



(57) Abstract: The present invention provides a method for altering a B cell mediated pathology in a patient. This method comprises administering a composition comprising at least one and/or two chimeric proteins. Each chimeric protein comprises at least a portion of either the V_H or V_L region of an immunoglobulin molecule from particular B cells from a patient having a B cell mediated pathology, and an immunoglobulin constant region. The genes encoding V_H and/or V_L regions and the genes encoding immunoglobulin constant regions are isolated and inserted into an expression vector. The chimeric proteins are produced by introducing the expression vectors into insect cell lines. The chimeric proteins are purified using antibody affinity columns, and then chemically conjugated to an immunogenic carrier, keyhole-limpet hemocyanin (KLH). Since the conjugates comprise chimeric proteins made specifically from particular B cells from a patient having B cell mediated pathology, when it is administered to such a patient, with or without a cytokine, such as granulocyte-macrophage-CSF, or a chemokine, it can induce immune responses to alter such a B cell mediated pathology.

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METHOD AND COMPOSITION FOR ALTERING A B CELL MEDIATED PATHOLOGY

RELATED APPLICATIONS

5 This application claims priority to the U.S. Provisional Application No. 60/224,723, entitled "Method for Producing an Idiotypic Vaccine," the U.S. Provisional Application No. 60/224,722 entitled "Expression Vectors for Production of Recombinant Immunoglobulin" and the U.S. Provisional Application No. 60/279,079 entitled "Method and Composition for Altering a B Cell Mediated Pathology."

10

FIELD OF THE INVENTION

 This invention relates generally to the field of immunology and immunotherapy. More specifically, this invention relates to methods and compositions for altering B cell mediated pathologies, such as B cell malignancies and/or autoimmune diseases.

15

BACKGROUND OF THE INVENTION

 The immune system produces both antibody-mediated and cell-mediated responses. Each type of immune response is regulated by a type of lymphocyte, B cells (for antibody-mediated response) and T cells (for cell-mediated response). B cells initially
20 recognize an antigen when the antigen binds to the IgM and IgD molecules on the B cell's surface. Each B cell clone recognizes only specific antigens due to the unique idiotype of that clone. Upon recognition of the antigen, B cells internalize and process the antigen for presentation via MHC class II molecules. B cells can thereby function as an antigen presenting cell ("APC") for T cells. T cells bind to portions of foreign proteins (antigens)
25 when portions of the protein associate with a major histocompatibility complex molecule ("MHC"), typically on an APC, in which the antigen is digested into fragments and presented on the surface of the APC bound to its MHC.

 Several types of cancers have their origin in the circulatory system. Among the major types are: leukemias, a neoplasm of the bone marrow and blood; myelomas, a

cancer of B cells; and lymphomas, a group of cancers that originate in the lymphatic system. Lymphomas can be further classified into several groups; one of these groups is the non-Hodgkin's lymphomas which, in turn, forms a diverse group of cancers. Three broad categories of these lymphomas are defined according to the International Working Formulation for tumor classification, low grade, intermediate grade and high grade, which differ in their curability and aggressiveness (Cheson, *et al.*, "Report of an International Workshop to Standardize Response Criteria for Non-Hodgkin's Lymphomas," *J.Clin Oncol.* 17(4):1244, 1999). Overall, these lymphomas collectively rank fifth in the United States in terms of cancer incidence and mortality, and approximately 50,000 new cases are diagnosed each year.

In a recent study which examined fifty-one case isolates of high-grade non-Hodgkin's lymphoma (NHL), forty-three were shown to be derived from B cells while eight were shown to be derived from T cells (Brown *et al.*, *Histopathology* 14:621-27, 1989). Therefore, treatments directed specifically towards pathological B cells would be valuable in the treatment of non-Hodgkin's lymphomas and myelomas.

Initial attempts in the field to develop an immunology-based treatment directed at antigens uniquely produced by malignant B cells involved laboriously isolating and purifying idiotypic (Id) proteins directly from the pathological B cells. This purified protein was first used in model systems to treat the associated lymphoma. It was demonstrated that this active immunization against idiotypic determinants on isolated proteins could produce resistance to tumor growth in a mouse model system (Daley *et al.*, *J.Immunol.* 120(5):1620-24, 1978; Sakato *et al.*, *Microbiol. Immunol.* 23(9):927-31, 1979). This phenomenon of resistance to tumor growth has been subsequently reproduced in a number of additional experimental tumor models (Stevenson *et al.*, *J.Immunol.* 130(2):970-03, 1983; George *et al.*, *J.Immunol.* 141(6):2168-74, 1988; Kwak, *et al.*, *Blood* 76(11):2411-17, 1990).

Among the first attempts at bringing this idea and technology into the clinic was very labor intensive and utilized mouse monoclonal antibodies generated against proteins isolated from the patients' individual lymphomas following biopsy. Meeker and coworkers generated mouse monoclonal anti-idiotypic antibodies for treatment of eleven

patients after most had already undergone conventional lymphoma therapy (Meeker *et al.*, *Blood* 65:1349-63, 1985). Positive results were obtained in roughly half the patients, with one case of apparent remission. In some of the patients, however, the lymphoma cells developed a resistance to the antibody via switching the class of cell surface-expressed antibodies (Meeker *et al.*, *N Engl J Med.* 312:1658-65, 1985).

Another way a B cell lymphoma clone developed resistance to anti-idiotypic antibodies is via a somatic mutation in the CDR2 region (Cleary *et al.*, *Cell* 44:97-106, 1986), thereby evading recognition. While this passive immunity approach for treatment has the advantage that it only requires isolation and purification of a relatively minor amount of idiotypic protein from a patient for raising an immune response in a mouse, the usefulness for treating lymphomas with monoclonal antibodies directed at idiotypes is limited. In the absence of a robust and convenient way to produce large quantities of idiotypic protein, however, this could prove to be the only practical way to exploit the abilities of the immune system to directly attack the idio type of a B cell lymphoma.

Kwak *et al.* pursued a different approach and attempted the active immunization of patients using proteins purified from their own unique lymphomas in spite of the logistical requirement for isolating large quantities of idiotypic proteins (Kwak *et al.*, *N. Engl. J. Med.* 327:1209-15, 1992). Patients who had minimal or no disease following chemotherapy were treated by vaccination with autologous idio type proteins. In order to obtain sufficient quantities of idiotypic proteins for vaccination, lymphoma cells obtained by biopsy were fused with an established cell line to facilitate their growth in tissue culture, and the secreted idio type proteins were purified via chromatography. Large scale application of this method of immunization is precluded due to the extreme labor requirements, technical barriers, and prohibitive costs. Additionally, concerns have recently been raised concerning the viral loads associated with protein production in mammalian cells.

In a following paper, Hsu *et al.* reported on the phase I/II of the above clinical trial utilizing vaccination of the idio type conjugated to keyhole limpet hemocyanin (KLH) in the treatment of B-cell lymphoma (Hsu *et al.*, *Blood* 89:3129-35, 1997). After standard chemotherapy, 41 patients with refractory non-Hodgkin's B-cell lymphoma were vaccinated with a tumor-specific idio type. As per Kwak *et al* (1992), *supra*, the tumor-

- specific idiotype antigens were obtained by chromatographic purification of proteins produced by the patients' hybridomas. These proteins were therefore composed of the entire variable and constant regions of the patient's own immunoglobulin from the patients' lymphomas. The results showed that the generation of an anti-idiotype response correlated with improved clinical outcome. The duration of freedom from disease progression and overall survival of all patients mounting an anti-idiotype cellular immune response were significantly prolonged compared to those patients who did not mount an immune response. This study confirms that patients with B-cell lymphomas can be induced to make a specific immune response against tumor idiotype (Id) protein.
- Furthermore, the ability to generate an anti-idiotype immune response correlates with a more favorable clinical outcome. However, to treat each individual patient, lymphoma cells obtained by biopsy must be fused to established cell lines in order to allow the production of sufficient protein to vaccinate a typical patient. This process would be difficult or impractical to use on a commercial scale.
- More recently, Bendandi *et al.* demonstrated idiotypic, patient-specific vaccination-induced remissions in patients with follicular lymphoma (Bendandi *et al.*, *Nat. Med.* 5:1171-77, 1999). Following standard chemotherapy, twenty patients demonstrating complete clinical remission were vaccinated using patient-specific idiotypic proteins accompanied by granulocyte-monocyte colony-stimulating factor (GM-CSF; *see infra.*).
- Molecular analysis of the translocations characteristic of this lymphoma was conducted prior to chemotherapy, at clinical remission and following vaccination therapy. Eight of eleven patients with detectable translocations after chemotherapy-induced remission were found to undergo complete molecular remission following this vaccination. Tumor-specific cytotoxic CD8⁺ and CD4⁺ T cells were found in 19 of 20 patients. Tumor-specific antibodies were also detected but were not found to be required for remission. Again, this study used idiotypic proteins made up of the entire variable and constant region of the immunoglobulin found associated with the patient's lymphoma and produced by heterohybridoma fusion.

Therefore, directing an immune response to the idiotype of cells is a promising approach, but the above techniques are limited by the requirement of producing sufficient quantities of idiotypic proteins from each patient's lymphoma cells.

The concept of anti-idiotypic immunity against B cell tumors has also been used in the case of multiple myeloma. Results have been reported by Kwak and coworkers regarding its use in enhancing the specific efficacy of allogeneic marrow grafts by pre-immunizing the donor with myeloma IgG isolated from the patient (Kwak *et al.*, *Lancet* 5 345 (8956):1016-20, 1995). Also, Massaia and coworkers vaccinated patients in remission following high-dose chemotherapy, followed by peripheral blood stem cell transplantation (Massaia *et al.*, *Blood* 94:673-83, 1999).

Granulocyte-monocyte colony-stimulating factor (GM-CSF), used above in Bendandi *et al.*'s study, is a hematopoietic growth factor which stimulates proliferation and differentiation of hematopoietic progenitor cells. This cytokine also plays a role in 10 shaping cellular immunity by augmenting T-cell proliferation (Santoli *et al.*, *J.Immunol.* 141(2):519-26, 1988). increasing expression of adhesion molecules on granulocytes and monocytes (Young *et al.*, *J.Immunol.* 145(2):607-15, 1990; Grabstein *et al.*, *Science* 232(4749):506-08, 1986), and augmenting antigen presentation (Morrissey *et al.*, 15 *J.Immunol.* 139(4):1113-9, 1987; Heufler *et al.*, *J. Exp. Med.* 167(2):700-05, 1988; Smith *et al.*, *J.Immunol.* 144(5):1777-82, 1990).

Cell-based vaccines genetically engineered to produce GM-CSF have been shown to induce cellular immune responses capable of eliminating systemic lymphomas in preclinical models. This effect is mediated exclusively through activation of the cellular 20 arm of the immune system (Levitsky *et al.*, *J. Immuno.* 156(10): 3858-65, 1996). Similarly, low doses of free GM-CSF have been shown to enhance the protective anti-tumor immunity induced by idiotypic protein-KLH immunization because of its ability to enhance immunity through an effect on the CD8 cells (Kwak *et al.*, *Proc. Natl. Acad. Sci. USA* 93(20):10972-77, 1996. In one study, GM-CSF was shown to be the best 25 immunomodulator to generate anti-tumor immunity among those tested in a model system (Dranoff, G., *Proc. Natl. Acad. Sci. USA* 90(8):3539-43, 1993.)

GM-CSF has also been used as a portion of a chimeric protein used to generate an immune response in model systems. Chen and Levy (Chen and Levy, *J. Immunol.* 154(7):3105-17, 1995; U. S. Patent No. 6,099,846) studied the production of mouse 30 monoclonal antibodies using a chimeric protein containing a portion of GM-CSF plus a portion of an antigen of interest, namely an idiotypic region obtained from a murine B-cell

tumor, 38C13, both fused to portions of human immunoglobulin chains. Chen and coworkers have also studied fusion proteins where the GM-CSF moiety has been replaced by portions of IL-2 or IL-4 (Chen *et al.*, *J.Immunol.* 153(10):4775-87, 1994). One explanation for the requirement of including the GM-CSF moiety (or interleukin moiety) was to augment the effect of low levels of chimeric protein produced by the mammalian cell expression system. However, the use of purified GM-CSF co-administered with a chimeric protein to enhance the immune response of a vaccination has not been demonstrated.

With the advent of recombinant DNA technology, heavy and light chain cDNA molecules can now be cloned from hybridomas or from combinatorial libraries employing the polymerase chain reaction (PCR). This recombinant DNA technology allows researchers to manipulate the effector function or the binding function of a selected monoclonal antibody. In addition, combinatorial libraries of immunoglobulins can be generated by cloning a large number of V_L and V_H genes, randomly assorting them to create a library of different binding specificities, expressing them in *E. coli*, then screening the stochastic library for clones with the desired binding affinities (Huse *et al.*, *Science* 246(4935):1275-81; 1989). Using this recombinant approach, human antibodies were cloned with high affinity and specificity for tetanus toxoid from a randomized combinatorial library expressed in *E. coli* (Mullinax *et al.*, *Proc. Natl. Acad. Sci.* 87(20):8095-99, 1990). The immunoglobulin genes were cloned from activated B-cells into bacteriophage vectors using the polymerase chain reaction (PCR) with specific primers. The H and L chains were randomly combined and co-expressed in *E. coli* to comprise a library of 10^7 members. This combinatorial library was screened with ^{125}I -tetanus toxoid and 0.2% of the clones displayed binding activity (Mullinax *et al.*, *supra*). In addition, murine monoclonal antibodies have also been identified using a similar approach (Huse *et al.*, *supra*; Caton *et al.*, *Proc. Natl. Acad. Sci.* 87(16):6450-54, 1990). Winter and co-workers used a plasmid vector to clone immunoglobulin domains by the polymerase chain reaction for expression in bacteria (Orlandi *et al.*, *Proc. Natl. Acad. Sci.* 86(10):3833-37, 1989).

Newly developed *E. coli* antibody cloning systems are very useful for the identification of genes encoding desired binding specificities. However, antibodies

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produced in *E. coli* are not generally useful for therapeutic applications. Typically, only the antibody antigen binding fragments, Fab or Fv, can be produced as secreted products in bacteria. In the rare instance when a whole chain tetrameric IgG has been produced in *E. coli*, the C_{H2} domains are not glycosylated. Nonglycosylated antibodies lack the

5 cytolytic activities antibody-directed cellular cytotoxicity (ADCC) and complement activation that make passive immunotherapy so powerful. Mammalian expression systems produce glycosylated antibody and thus circumvent this limitation of the bacterial system. However, recent modifications in the CBER division of the FDA's "Points to Consider" clearly signal their concerns about viral loads associated with monoclonal antibodies

10 produced in mammalian cells. Moreover, it is expected that any engineered antibody produced in a mammalian expression system will be quite expensive (\$1500-\$5000 per dose). Alternative expression systems that circumvent the difficulties encountered with current mammalian and bacterial systems are therefore highly desirable.

The baculovirus expression system is an attractive alternative to antibody

15 production in *E. coli* and mammalian cells. The expression of recombinant proteins using the baculovirus system has been demonstrated in the past several years and has emerged as an excellent choice for high yield production (1-100 mg/L) of biologically active proteins in eukaryotic cells. The baculovirus/insect cell system also circumvents the solubility problems often encountered when recombinant proteins are overexpressed in prokaryotes.

20 In addition, insect cells contain the eukaryotic post-translational modification machinery responsible for correct folding, disulfide formation, glycosylation, β -hydroxylation, fatty acid acylation, prenylation, phosphorylation and amidation not present in prokaryotes. The production of a functional, glycosylated monoclonal antibody recognizing human colorectal carcinoma cells from a baculovirus expression system has been recently

25 demonstrated (Nesbit, *J. Immunol. Methods* 151:201-208, 1992). Additionally, expression of recombinant IgA has also been demonstrated in baculovirus cells, and this IgA was correctly assembled into heavy chain/light chain heterodimers, N-glycosylated, and secreted (Carayannopoulos *et al.*, *Proc. Natl. Acad. Sci.* 91:8348-52, 1994, PCT Publication No. WO 98/30577, U.S. Patent No. 6,063,905). However, the use of

30 baculovirus to express a chimeric idiotypic protein for use as an immunotherapeutic agent

to modify a B cell pathology such as B cell malignancies and autoimmune diseases has not been demonstrated.

SUMMARY OF THE INVENTION

5 The present invention provides a method for altering a B cell mediated pathology in a patient. This method includes administering a composition that contains at least one chimeric protein having at least a portion of a V_H or V_L region of an immunoglobulin variable region and at least a portion of an immunoglobulin constant region. The V_H or V_L region used in this composition is associated with a particular immunoglobulin produced
10 by a B cell from a patient having a B cell mediated pathology. After administering such a composition into a patient, the B cell mediated pathology in the patient is altered.

 The present invention also provides a method for altering a B cell mediated pathology in a patient by administering a composition containing two different chimeric proteins. Each chimeric protein has at least a portion of a V_H and/or V_L region of an
15 immunoglobulin chain linked to at least a portion of an immunoglobulin constant region. The V_H and/or V_L regions that are part of the chimeric protein are associated with particular immunoglobulin chains from a B cell of the patient having a B cell mediated pathology.

 Specific immunoglobulin chains containing patient-derived unique V_H and/or V_L
20 chains can be developed as therapeutic compositions. They will have therapeutic value for patients suffering from a variety of B cell malignancies or autoimmune diseases. Among the major types of cancers that can be treated are leukemias, myelomas, and lymphomas; among the lymphomas is non-Hodgkin's lymphoma. As an example of the therapeutic value of the instant invention, antigens derived from B cell lymphomas have been used to
25 treat patients.

 Suspected self-antigens can be used to affinity purify B cells involved in autoimmune diseases, such as multiple sclerosis (MS) (Warren and Catz, *Mult. Scler.* 6(5):300-11, 2000), systemic lupus erythematosus (SLE) (Zhang, J. *et al.*, *J. Immunol.* 166(1):6-10, 2001; Odendahl, M. *et al.*, *J. Immunol.* 165(10):5970-79, 2000), anti-Hu
30 associated paraneoplastic neurological syndromes (Rauer, S. and Kaiser, R., *J. Neuroimmunol.* 111(1-2):241-44, 2000); and autoimmune hepatitis (AIH) (Ogawa, S. *et*

al., *J. Gastroenterol. Hepatol* (1):69-75, 2000). Other autoimmune diseases which may have B cell involvement include rheumatoid arthritis (RA), myasthenia gravis (MG), autoimmune thyroiditis (Hashimoto's thyroiditis), autoimmune uveoretinitis, polymyositis, scleroderma, and certain types of diabetes. Following the purification of a small number
5 of pathogenic B cells, the variable portion of the immunoglobulins expressed by these cells may be cloned via PCR using the methods described in the invention. Once cloned, the V_H and/or V_L portions of the immunoglobulin chains specifically involved in the B cell pathology can be used to make chimeric proteins which can be expressed in a baculovirus system as described herein.

10 The immunoglobulin constant regions used in the above compositions and chimeric protein can be from IgG₁, IgG₂, IgG₃, IgG₄, IgA₁, IgA₂, IgM, IgD, IgE heavy chains, and κ or λ light chains or portions thereof. In some of the embodiments, the chimeric protein only contains either the V_H and/or V_L region of an immunoglobulin region with an immunoglobulin constant region. Examples of chimeric proteins include
15 V_H -IgG_{γ1}, V_L - κ , and V_L - λ . In another embodiment, the composition contains two chimeric proteins that each respectively contains a V_H and V_L region with an immunoglobulin constant region. Examples include V_H -IgG_{γ1} and V_L - κ , and V_H -IgG_{γ1} and V_L - λ .

The present invention also provides a method for producing chimeric proteins
20 using recombinant DNA technology and an expression system. This method includes the following steps: (a) isolating genes encoding the V_H or V_L region of an immunoglobulin chain from B cells of a patient having a B cell mediated pathology, (b) inserting the isolated gene encoding the V_H or V_L region of an immunoglobulin chain and the gene encoding an immunoglobulin constant region into an expression vector to allow the
25 expression of a chimeric protein, (c) producing the chimeric protein by introducing the expression vector into insect cell lines and allowing its expression, and (d) isolating the chimeric protein. The method for producing chimeric proteins further includes a step of inserting a gene encoding either the V_H and/or V_L region of an immunoglobulin chain and a gene encoding a second immunoglobulin constant region into the expression vector to
30 allow the expression of the second chimeric protein.

The present invention further provides a composition for altering a B cell mediated pathology in a patient. This composition contains at least one chimeric protein having at least a portion of a V_H and/or V_L region of an immunoglobulin chain and at least a portion of an immunoglobulin constant region. In preferred embodiments, the chimeric proteins
5 may comprise at least a portion of a V_H region of an immunoglobulin chain and at least a portion of an immunoglobulin constant region. The V_H or V_L region that is part of the chimeric protein are associated with a particular immunoglobulin chain from a B cell of a patient having a B cell mediated pathology. The composition further contains a second chimeric protein having at least a portion of a V_H and/or V_L region of an immunoglobulin
10 chain and at least a portion of a second immunoglobulin constant region. In other preferred embodiments, the second chimeric protein may comprise at least a portion of a V_H or V_L region of an immunoglobulin chain and at least a portion of an immunoglobulin constant region. The V_H or V_L region that is part of the chimeric protein is associated with a particular immunoglobulin chain from a B cell of a patient having a B cell mediated
15 pathology.

In one of the embodiments of the invention, the composition comprises two chimeric proteins. The first of the chimeric protein comprises the entire V_H region and a human constant region of an immunoglobulin $IgG_{\gamma 1}$ (V_H - $IgG_{\gamma 1}$), and the second chimeric protein comprises the entire V_L and a human κ or λ constant region (V_L - C_{κ} or V_L - C_{λ}). In
20 other preferred embodiments, either or both of the chimeric proteins may comprise at least a portion of a V_H and/or V_L region of an immunoglobulin chain, plus a linker region, and at least a portion of an immunoglobulin constant region.

In another embodiment of the invention, the composition contains a single chimeric protein containing either a V_H and/or V_L region from a particular
25 immunoglobulin chain from a B cell of a patient and an immunoglobulin constant region. Examples include chimeric proteins V_H - $IgG_{\gamma 1}$, V_L - κ , V_L - λ , V_L - $IgG_{\gamma 1}$, V_H - κ , and V_H - λ .

In one of the embodiments of the invention, the expression vector used to express the chimeric proteins is a baculovirus vector. The vector preferably contains two expression cassettes each having a promoter, a secretory signal sequence and a chimeric
30 protein. One expression cassette contains the baculovirus AcNPV p10 promotor linked to

the honey bee melittin secretory signal sequence. The other expression cassette has the polyhedrin promotor linked to a human placental alkaline phosphatase secretory signal sequence. In addition to the listed promoters and signal sequences, other promoters and signal sequences known to those skilled in the art could be used. In some preferred
5 embodiments, the signal sequences are endogenous signal sequences associated with the V_H and V_L genes isolated from patients, or other signal sequences involved in antibody production. The genes encoding the V_H or V_L portions of the immunoglobulin chains, and the genes encoding immunoglobulin constant region are inserted, separately and/or together, into the above expression cassette of the baculovirus vector allowing expression
10 of one or two chimeric proteins. In a preferred embodiment, the constant region of the immunoglobulin heavy chain, such as $IgG_{\gamma 1}$, with either the V_H or V_L region, is controlled by the polyhedrin promotor.

Chimeric proteins produced are purified using affinity columns with anti-immunoglobulin antibodies or Ig-binding proteins, such as protein A for the constant
15 region of an immunoglobulin heavy chain, and protein L for kappa light chains, and/or any other proteins that bind to an immunoglobulin binding domain.

The present invention also contemplates covalently coupling the chimeric proteins to a carrier protein such as keyhole limpet hemocyanin (KLH). The composition of the present invention may also be administered into a patient together with a cytokine such as
20 granulocyte-macrophage-CSF (GM-CSF), or a chemokine such as a monocyte chemotactic protein 3 (MCP 3). Because the present composition of the present invention containing chimeric protein(s) is specifically related to a particular immunoglobulin from B cells of a patient having B cell mediated pathology, administration of this composition induces an immune response against the disease specific idiotype in which particular V_H
25 or V_L segments are involved. Similarly, responses against B cells associated with autoimmune diseases involving B cells that use a restricted repertoire of immunoglobulin V-region segments, such as V_H or V_L segments may induce a therapeutic result. Thus, the administration of the composition of the present invention alters a B cell mediated pathology and/or autoimmune diseases in a patient. The administration routes for the
30 invented composition include but are not limited to oral delivery, inhalation delivery, injection delivery, transdermal delivery, and the like.

All U.S. patents and applications; foreign patents and applications; scientific articles; books; and publications mentioned herein are hereby incorporated by reference in their entirety, including any drawings, figures and tables, as though set forth in full.

5

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: A general scheme for producing a composition comprising chimeric proteins for V_H or V_L regions of a particular immunoglobulin from B cells from a patient having B cell mediated pathology.

Figure 2: Plasmid map of a baculovirus expression vector p2Bac with
10 multiple cloning sites.

Figure 3: DNA sequence of baculovirus expression vector p2Bac (SEQ ID NO:5). The sequence is depicted from 5' to 3'. The p2Bac vector contains the AcNPV polyhedrin gene promoter (nucleotides 1 to 120 of the GenBank accession number X06637 (SEQ ID NO:92)) and the AcMNPV p10 promoter (nucleotides 8 to 237 of
15 GenBank accession number A28889 (SEQ ID NO:93)).

Figure 4: DNA sequence of the plasmid pTRABac/9F12. This plasmid contains the genes for the heavy and light (κ) chains expressed by the stable human cell-line 9F12. This cell line produces a human IgG1/ κ antibody specific for tetanus toxoid (SEQ ID NO:89). The underlined regions represent sequences encoding mature 9F12
20 IgG₁ (TTTACCC....) and kappa (ATCGACA...) chains, respectively. The sequence is depicted from 5' to 3'.

Figure 5a: Plasmid map of recombinant baculovirus expression vector pTRABacHuLC κ HC γ_1 with IgG γ_1 constant regions.

Figure 5b: Plasmid map of recombinant baculovirus expression vector
25 pTRABacHuLC λ HC γ_1 with IgG γ_1 constant regions.

Figure 6A: DNA sequence of pTRABacHuLC κ HC γ_1 (SEQ ID NO:6). The sequence is depicted from 5' to 3'.

Figure 6B: DNA sequence of pTRABacHuLC λ HC γ_1 (SEQ ID NO:7). The sequence is depicted from 5' to 3'.

Figure 6C: DNA sequence of pTRABacHuLC_κHC_{γ1} following modification utilizing the kappa stuff primers (SEQ ID NO:90). The sequence is depicted from 5' to 3'.

Figure 6D: DNA sequence of pTRABacHuLC_λHC_{γ1} following modification utilizing the lambda stuff primers (SEQ ID NO:91). The sequence is depicted from 5' to 3'.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The possibility of evoking an immune response that would recognize and eliminate neoplastic cells while sparing normal tissue represents an exciting approach to the treatment of cancer. Inducing such an immune response is assisted by identifying a unique tumor antigen. B-cell malignancies express a unique antigen, the immunoglobulin idiotype (Id), on their surface. This antigen contains protein sequences from both the variable immunoglobulin heavy and light regions (V_H and V_L). Each B-cell harbors a unique genetic sequence used in the production of the immunoglobulin idiotype. Consequently, as most B cell malignancies arise from the clonal expansion of a single B cell, all cells comprising a B-cell malignancy expresses a unique Id protein. Hence, idiotypic protein should serve as an ideal target for immune-based therapy of any B cell malignancy, such as lymphoma or leukemia.

Passive Immunotherapy

Early immunotherapy strategies focused on the use of monoclonal antibodies against tumor-specific idiotype (anti-Id MoAb). This approach resulted in tumor regression and long-lasting remissions in several patients with non-Hodgkin's lymphoma. However many patients experienced eventual relapse (Miller *et al.*, *N. Engl. J. Med.* 306(9):517-22, 1982; Maloney *et al.*, *Blood* 80(6):1502-10, 1992; Brown *et al.*, *Blood* 73(3):651-61, 1989; Brown *et al.*, *Semin. Oncol.* 16(3):199-210, 1989; Meeker *et al.*, *Blood* 65(6):1349-63, 1985).

One difficulty that arose in the studies described above was that cells of a malignant B or T cell lymphoma could alter their expression of their idiotypic immunoglobulins or T cell receptors. Two examples of this were described in some of the

articles listed above (Cleary *et al.*, 1986, and Meeker *et al.*, 1985). It was also shown that T cell leukemia cells could escape anti-idiotypic antibodies by reducing their expression of surface T-cell receptor (Maecker *et al.*, *J. Immunol.* 141:2994-3002, 1985), and this was confirmed for B cell leukemias in an animal model (Stevenson *et al.*, *J. Immunol.*

- 5 130(2):970-3, 1983). Other studies demonstrated that there is idiotypic variation even within a given human B cell lymphoma (Berinstein *et al.*, *J. Immunol.* 144(2):752-8, 1990; Levy S, *et al.*, *J. Exp. Med.* 168(2):475-89, 1988;). Such mutations appeared responsible for the decreased effectiveness of the anti-Id MoAb over time (Berinstein *et al.*, *supra*; Tao *et al.*, *Nature* 362(6422):755-8, 1993; Chen *et al.*, *J. Immunol.* 153(10):4775-87, 10 1994).

- One way to avoid this problem is via the generation and use of a polyclonal antisera against the idiotypic protein. Caspar and co-workers studied the potential of a polyclonal antibody-based therapy in a mouse model system (Caspar *et al.*, *Blood* 90:3699-706, 1997). These authors vaccinated a mouse using idiotypic proteins from a 15 non-Hodgkin's leukemia patient who had relapsed following successful monoclonal antibody therapy. The resultant polyclonal antibodies recognized idiotypic proteins from both the original tumor and all variants. Therefore, generation of polyclonal antibody response specific to the idioype of a B cell lymphoma or leukemia would represent an improvement over monoclonal antibody therapy. Producing sufficient quantities of 20 protein to for a vaccination to produce polyclonal antibodies is a significant burden of this approach.

Active Immunotherapy

- Active immunotherapy may avoid the phenomenon of mutational escape seen with 25 passive immune strategies. Such therapy has the potential to generate a broader immune response and thereby recognize the heterogeneous tumor cell population that can arise over time. The difficulty with active immunotherapy lies in convincing the patient's immune system to react against a perceived "self antigen" expressed by the tumor. As with idiotypic protein, many of the antigens expressed by tumors are weak immunogens.

In the instant invention, the unique specificity of the immune system has been adapted to treat B cell malignancies. In the instant invention, the DNA sequence encoding the variable region of the idiotypic immunoglobulins was cloned using primers derived from the 5' end of each unique subfamily of light and heavy immunoglobulin chains together with a constant region primer. Typically, this process uses one of several suitable cloning techniques such as PCR. These constant region primers, in combination with one for the V_H region and one for the V_L region, may be used to clone the variable regions as a first step in producing a chimeric protein comprising a variable region and a constant region. Alternatively, techniques such as 5' RACE may be used. In the case of one patient described *infra*, 5' RACE was used to clone the variable regions of the heavy and light immunoglobulin chains in order to produce a chimeric protein. Examples of chimeric proteins include: V_L / C_K , V_L / C_λ , $V_L / IgG_{\gamma 1}$, $V_H / IgG_{\gamma 1}$, V_H / C_K , and V_H / C_λ . These chimeric proteins are produced in insect cells using a baculovirus vector. The chimeric protein thus comprises a portion of a variable region from an immunoglobulin molecule from a patient and also comprises a portion of a constant region from a source other than the patient. In preferred embodiments, the heavy and light chain constant regions are derived from 9F12 cells. However, other sources for immunoglobulin constant region genes may be used. These chimeric proteins are predicted to be more efficiently produced than using existing systems for producing idiotypic proteins and will be excellent immunogens for use in vaccination protocols.

The present invention fills the great demand for an effective treatment for B cell mediated pathologies and autoimmune diseases. The inventions take advantage of the unique cell surface antigens present on the surface of B cells involved in B cell pathologies, and are prepared in a patient-specific manner. Such vaccines provide exquisite selectivity by being tailored to the markers unique to the pathogenic B cells found in a given patient.

The novel baculovirus/insect cell expression system has proven effective for the efficient production of functional antibodies for immunotherapy from any given patient. This baculovirus expression vector was designed such that only two custom gene-specific primers were needed to amplify any pair of antibody variable regions for easy subcloning

and expression as human kappa light chain and IgG γ_1 heavy chain. The incorporation of heterologous secretory signal sequences, which directed the heavy and light chains to the secretory pathway, were incorporated for the expression of large amounts of active immunoglobulin from insect cells. This vector should be useful for the expression of any kappa light chain variable region (V_L) in frame with human kappa constant region and secreted via the human placental alkaline phosphatase secretory signal sequence; and any heavy chain variable region (V_H) in frame with the human IgG γ_1 constant domain led by the honey bee melittin secretory signal sequence. In other systems, the lambda light chain constant region replaces the kappa constant region. The chimeric protein is then expressed with the V_L region in frame with human lambda constant region and secreted via the human placental alkaline phosphatase secretory signal sequence, along with any heavy chain variable region (V_H) in frame with the human IgG γ_1 constant domain led by the honey bee melittin secretory signal sequence. Any monoclonal antibody, mouse or human, either from a monoclonal cell line or identified by phage display cloning, could be easily expressed as whole human IgG γ_1 / κ or IgG γ_1 / λ in this vector after two simple subcloning steps. Additionally, different immunoglobulin types, including IgG γ_2 , IgG γ_3 , IgG γ_4 , IgA, IgA, IgA₁, IgA₂, IgM, IgD, IgE heavy chains, or segments thereof, could be used in place of IgG γ_1 . Furthermore, besides those signal sequences described *supra*, the instant invention may use other secretory signal sequences such as the endogenous secretory sequences associated with the immunoglobulin genes derived from a given patient. Additionally, one of skill in the art would be able to select several different primers that could be used equivalently in this system to produce equivalent results to amplify any pair of antibody variable regions for easy subcloning.

In some instances, utilization of the baculovirus system for the expression of biologically active proteins has been hampered by the inability to efficiently solubilize recombinant proteins without excessive proteolytic degradation. In order to circumvent solubility and proteolysis problems encountered with the expression of recombinant proteins in insect cells, baculovirus transfer vectors were developed for the efficient secretion of biologically active proteins. These vectors that facilitate the secretion of recombinant proteins from host insect cells are constructed by inserting functional

secretory leader sequences downstream of the polyhedrin promoter. In-frame insertion of cDNA sequences resulted in the synthesis of proteins containing a heterologous signal sequence which directed the recombinant protein to the secretory pathway. Human and insect leader sequences were both tested to maximize secretion of heterologous proteins

5 from insect cells. The human placental alkaline phosphatase signal sequence (SEQ ID NO:1: MLGPCMLLLLLLLGLRLQLSLG; DNA sequence is SEQ ID NO:2: ATG GTG GGA CCC TGC ATG CTG CTG CTG CTG CTG CTA GGC CTG AGG CTA CAG CTC TCC CTG GGC) and the honeybee melittin signal sequence (SEQ ID NO:3: MKFLYNVALVFMVVYISYIYA; DNA sequence is SEQ ID NO:4: ATG AAA TTC

10 TTA GTC AAC GTT GCA CTA GTT TTT ATG GTC GTG TAC ATT TCT TAC ATC TAT GCG) have both proved useful for the secretion of numerous bacterial and human proteins (Mroczkowski *et al.*, *J Biol. Chem.* 269:13522-28, 1994 and Tessier *et al.*, *Gene* 98:177-83, 1991).

To tailor the present invention to a particular patient first requires identification

15 and isolation of the genes encoding the unique antigens, and then the means of producing those antigens. This may be accomplished in a number of different ways available to one of skill in the art. For example, a recently developed method that is adapted to the needs of the instant invention uses a novel baculovirus/insect cell expression system and was recently developed for the efficient production of functional antibodies for immunotherapy

20 (see U.S. Provisional Application Serial No. 60/244,722, entitled "Expression Vectors for Production of Recombinant Immunoglobulin").

Expression of recombinant proteins using the baculovirus system allows the production of large quantities of biologically active proteins without many of the drawbacks associated with proteins made in bacteria, and also avoids the complications of

25 using mammalian cells. For example the immunoglobulin genes from the stable human cell-line 9F12 (ATCC#HB8177), which produces a human IgG1/ κ antibody specific for tetanus toxoid, were cloned into a baculovirus dual promoter expression transfer vector. Intact IgG1/ κ immunoglobulin was produced in insect cells that behaved similarly to the mammalian antibody in SDS-PAGE analysis and Western blots. The antibody produced

30 by insect cells was glycosylated. The binding affinities of purified Mab9F12 and purified

baculovirus expressed antibody were determined to be identical and production levels were determined to be approximately 5-10 µg/ml.

Soluble human immunoglobulin fragments containing specific epitopes of the particular variable regions can be produced in insect host cells via genetic engineering.

- 5 These soluble recombinant immunoglobulin proteins containing patient-derived particular V_H and/or V_L regions can be used as a therapeutic composition. When administered into the patient, it would specifically induce, in vivo, a cell mediated immune response for altering the B cell mediated pathology.

- 10 This technology has also been applied towards the rapid identification and cloning of patient-specific V_α and V_β genes expressed by a T cell lymphoma, then expressing these as recombinant κ/V_α or $IgG_{\gamma 1}/V_\beta$ molecules in insect cells (see U.S. Provisional Application Serial No. 60/266,133 entitled "Method and Composition for Altering a T Cell Mediated Pathology"). Molecules produced by this method were formulated and used to induce anti-idiotypic cell-mediated immunity against lymphomas in a patient-specific fashion.

- 20 The term "altering" or "alters" refers to the ability of a compound or composition of the invention to modulate a B cell mediated pathology. A compound which alters a B cell pathology may do so by a number of potential mechanisms, including raising antibodies directed at the compound which in turn destroys cells of the B cell pathology, inducing apoptosis in the B cells involved in the pathology, inhibiting further growth and division of cells of the B cell pathology, inducing cell-mediated immunity directed at the cells of the B cell pathology, or otherwise inhibiting the activity of the pathological B cells. The exact mechanism that causes the alteration need not be determined, but only that an alteration in the B cell mediated pathology occurs by some mechanism as a consequence of adding the inventive molecules or compositions.

- 25 The term "B cell mediated pathology" or "B cell pathology" refers to those diseases and conditions that arise from inappropriate replication or activity of B cells. In preferred embodiments, the B cell mediated pathology is a B cell lymphoma that results from inappropriate replication of B cells. B cell lymphomas are difficult to treat effectively with the currently available medical methods. Other types of B cell

pathologies which involve inappropriate replication of B cells include chronic and acute B cell leukemias, multiple myelomas, and some non-Hodgkin's lymphomas. Other preferred embodiments include a growing number of human diseases that have been classified as autoimmune disease, where the host's own immune system attacks the host's own tissue, such as multiple sclerosis (MS) (Warren and Catz, *Mult. Scler.* 6(5):300-11, 2000), systemic lupus erythematosus (SLE) (Zhang, J. *et al.*, *J. Immunol.* 166(1):6-10, 2001; Odendahl, M. *et al.*, *J. Immunol.* 165(10):5970-79, 2000), anti-Hu associated paraneoplastic neurological syndromes (Rauer, S. and Kaiser, R., *J. Neuroimmunol.* 111(1-2):241-44, 2000); autoimmune hepatitis (AIH) (Ogawa, S. *et al.*, *J. Gastroenterol. Hepatol* (1):69-75, 2000). Other candidate autoimmune diseases for treatment by the present invention include rheumatoid arthritis (RA), myasthenia gravis (MG), autoimmune thyroiditis (Hashimoto's thyroiditis), Graves' disease, inflammatory bowel disease, autoimmune uveoretinitis, polymyositis, scleroderma, and certain types of diabetes. The present treatments for these autoimmune diseases do not cure the disease, but instead only ameliorate the symptoms.

The term "B cell" refers to a cell of the immune system of an organism which is involved in the humoral immunity in normal functioning of a organism (i.e., one that is not experiencing a B cell mediated pathology). B cells are white blood cells that develop from bone marrow and produce antibodies; they are also known as B lymphocytes. In general, B cells are cells involved in antibody production in an organism.

The term "pathology" refers to a state in an organism (e.g., a human) which is recognized as abnormal by members of the medical community. The pathology to be treated in the present invention is characterized by an abnormality in the function of B cells.

The term "patient" refers to an organism in need of treatment for a pathology, or more specifically, a B cell pathology. The term refers to a living subject who has presented at a clinical setting with a particular symptom or symptoms suggesting the need for treatment with a therapeutic agent. The treatment may either be generally accepted in the medical community or it may be experimental. In preferred embodiments, the patient is a mammal, including animals such as dogs, cats, pigs, cows, sheep, goats, horses, rats, and mice. In further preferred embodiments, the patient is a human. A patient's diagnosis

can alter during the course of disease progression, either spontaneously or during the course of a therapeutic regimen or treatment.

An "organism" can be a single cell or multi-cellular. The term includes mammals, and, most preferably, humans. Preferred organisms include mice, as the ability to treat or
5 diagnose mice is often predictive of the ability to function in other organisms such as humans. Other preferred organisms include primates, as the ability to treat or diagnose primates is often predictive of the ability to function in other organisms such as humans.

The term "chimeric protein" refers to a protein which comprises a single polypeptide chain comprising segments derived from at least two different proteins. The
10 segments of the chimeric protein must be derived from heterologous proteins, that is, all segments of the chimeric polypeptide do not arise from the same protein. The chimeric proteins of the present invention include proteins containing portions of the V_H or V_L region of an immunoglobulin chain, but do not comprise the entire C region of those chains as found in the B cell clone from which the V_H or V_L regions is derived.
15 Furthermore, the V_H or V_L region may not include the entire variable region, but does include enough to generate an immune response. Chimeric proteins of the present invention may also include proteins in which a segment of the naturally occurring protein has been replaced with an equivalent naturally or non-naturally occurring segment. This includes replacing the IgG₁ constant region derived from a patient with the IgG₁ constant
20 region from a different source and would also include immunoglobulin constant regions in which a segment of the protein has been replaced with a linker, segment or domain that is partially or entirely manmade. In all cases, however, the gene for the chimeric protein of the instant invention will not be the same as the gene for the immunoglobulins which occur naturally in the patient. The gene for the chimeric protein will be distinguishable
25 from naturally occurring protein for one of the following reasons: (1) it will not be the full length immunoglobulin gene or cDNA from the patient, (2) it will be a different subtype than isolated from the patient, or (3) the nucleic acid sequence encoding the patient's IgG₁ constant region will differ from the IgG₁ gene used in the expression vector.

The terms "protein," "polypeptide," and "peptide" are used herein interchangeably.

The term "naturally" or "native" refers to a protein as it is isolated from nature. Thus, a naturally occurring protein may refer to a protein as it is found in nature which is encoded by a gene that has not been modified by the use of recombinant techniques. A native protein may refer to a protein as it may be found or synthesized in nature. These
5 terms may also apply to proteins which are produced by biological system such as the baculovirus virus system of the present invention or by the culture of cells derived from patients. A native protein may alternately refer to an isolated protein which has not been denatured. The term "native" may also refer to the manner in which polypeptide or protein is folded, either alone or in combination with other polypeptides, so that it
10 resembles similar proteins found in nature, or how it is modified after translation ("post-translational modifications") so that it resembles similar proteins found in nature. A naturally-occurring protein may be found only in pathological B cells from a single patient, nevertheless, this may be considered a naturally-occurring protein.

The term "segment" or "portion" is used to indicate a polypeptide derived from the
15 amino acid sequence of the proteins used for the chimeric proteins having a length less than the full-length polypeptide from which it has been derived. It is understood that such segments may retain one or more characterizing portions of the native polypeptide. Examples of such retained characteristics include: binding with an antibody specific for the native polypeptide, or an epitope thereof.

20 The terms " V_H " and " V_L " refer to the variable regions of the polypeptide chains of immunoglobulin molecules, or nucleic acids encoding such polypeptide chains. One skilled in the art realizes the meaning of these terms. The exact sequence of a variable region cannot be predicted and must be determined by isolating the sequence in question. The V_H and V_L regions isolated from particular patients are used in the instant invention.
25 The exact sequence of a kappa (κ) or lambda (λ) light chain is determined by clonal rearrangements of the V regions, J regions and Constant region of the light chain locus. (The kappa and lambda loci are separate and distinct.) The exact sequence of a heavy chain is determined by clonal rearrangements of the V regions, D regions, J regions and Constant region of the heavy chain locus. Additional sequence variation in the variable
30 region arises from imprecision during the recombination process and also is generated by somatic mutations subsequent to the end of the recombination process. The terms " V_H "

and "V_L" also refer to portions or segments of the V_H and V_L regions. A segment of the V_H and V_L region may also include all or substantially all of the V region. The term "substantially all" refers to approximately 90% of the entire variable region, or approximately 80% of the entire variable region. The portion of the V_H and V_L region present must be sufficient to allow the chimeric molecule to operate in the present invention. The terms "V_H" and "V_L" also refer to functional derivatives of such polypeptide regions as described *infra*.

The term "immunoglobulin constant region" refers to all or part of that portion of immunoglobulin molecules which are not encoded by the variable regions of immunoglobulins. The term "immunoglobulin constant region" may also refer to the DNA sequence encoding the immunoglobulin constant region. The immunoglobulin constant region includes the segments C_L, C_{H1}, C_{H2}, C_{H3}, and the Hinge region. Immunoglobulin types include IgG_{γ1}, IgG_{γ2}, IgG_{γ3}, IgG_{γ4}, IgA₁, IgA₂, IgM, IgD, IgE heavy chains, and κ or λ light chains or segments thereof. Any immunoglobulin constant region segments may be used in the instant invention, provided that the segment allows the immunoglobulin constant region to operate for the purposes of the present invention, for example, or the affinity purification of the chimeric molecule, via binding to Protein G, Protein A, Protein L, or appropriate antibody. Functional derivatives of the immunoglobulin constant region segments, as described *infra*, may also be used.

The term "immunoglobulin fold" or "immunoglobulin domain" refers to a structural element of the immunoglobulin super family. The immunoglobulin domain is a conserved, repeating structural domain of approximately 110 amino acids each.

Immunoglobulin domains are found in many protein molecules, including antibodies, the T cell antigen receptor, cytokine receptors (*e.g.*, the platelet-derived growth factor receptor with 5 Ig domains), cell adhesion molecules (*e.g.*, ICAM-1/CD54), and many others. Two immunoglobulin domains are found in each TCR; one in the variable region and one in the constant region. Two immunoglobulin domains are found in antibody light chains and four are found in IgG heavy chains. The present invention contemplates the replacement of one or two domains of the constant region with domains from a different molecule, such as an immunoglobulin molecule, to produce a modified

(chimeric) constant region which may have different properties such as binding to other molecules.

The terms "IgG₁, IgG₂, IgG₃, IgG₄, IgA, IgA₁, IgA₂, IgM, IgD, IgE" refer to classes and subclasses of human immunoglobulins. The terms may refer to either the DNA
5 sequences or the amino acid sequences of the proteins. The class and subclass of an immunoglobulin molecule is determined by its heavy chain. IgG and IgD are different classes of immunoglobulins; IgG₁ and IgG₂ are different subclasses of immunoglobulin molecules. The term "IgA" may refer to any subclass of IgA molecules. In preferred
10 embodiments, it refers to an IgA₁ molecule. In other preferred embodiments, it refers to an IgA₂ molecule. In some embodiments, the immunoglobulin heavy chain used may be a chimeric protein that contains amino acids from a second protein.

The term "IgG_H" refers to the heavy chain associated with the IgG₁ class of immunoglobulins. IgG₁ represents approximately 66% of human IgG immunoglobulins (Roitt *et al.*, *Immunology*, Mosby, St. Louis, pg. 4.2, 1993).

15 The terms "kappa constant region," "lambda constant region," "κ constant region," and "λ constant region" refer to the constant regions of kappa (κ) and lambda (λ) light chains that remain constant during the development of the immune system. The terms may refer to either the DNA sequences or the amino acid sequences of the proteins. In some embodiments, portions of the immunoglobulin light chain may be comprised in a
20 chimeric protein that contains amino acids from one or more other proteins.

The term "administering" relates to a method of contacting a compound with or into cells or tissues of an organism. The B cell mediated pathology can be prevented or treated when the cells or tissues of the organism exist within the organism or outside of the organism. Cells existing outside the organism can be maintained or grown in cell culture
25 dishes. For cells harbored within the organism, many techniques exist in the art to administer compounds, including (but not limited to) oral, parenteral delivery, dermal application, injection, and aerosol applications.

The B cell mediated pathology can also be prevented or treated by administering a compound of the invention, or an antibody raised to a compound of the invention, to B
30 cells displaying the characteristics of a pathology. The effect of administering a compound on organism function can then be monitored. The organism is preferably a

mouse, rat, rabbit, guinea pig, or goat, more preferably a monkey or ape, and most preferably a human.

The term "composition" refers to a mixture that contains the protein of interest. In preferred embodiments, the composition may contain additional components, such as
5 adjuvants, stabilizers, excipients, and the like.

The term "associated with" in reference to the relation of a variable region to a B cell clone refers to the variable region that is found on the immunoglobulins produced by a particular B cell clone.

The term "B cell clone" refers to the clonal descendants of a single B cell. Clonal
10 descendants of B cells express the same idiotype in the produced antibodies as the parental cell. One skilled in the art realizes that clonal descendants of a B cell may have undergone somatic mutation within the variable region of the immunoglobulin gene but still remain part of the B cell clone.

The term "isolating" refers to removing a naturally occurring nucleic acid sequence
15 from its normal cellular environment. Thus, the sequence may be in a cell-free solution or placed in a different cellular environment. The term does not imply that the sequence is the only nucleotide chain present, but that it is essentially free (about 90 - 95% pure at least) of non-nucleotide material naturally associated with it, and thus is distinguished from isolated chromosomes. Also, by the use of the term "isolating" in reference to
20 nucleic acid is meant that the specific DNA or RNA sequence is increased to a significantly higher fraction (2- to 5-fold) of the total DNA or RNA present in the solution of interest than in the cells from which the sequence was taken. This could be caused by a person by preferential reduction in the amount of other DNA or RNA present, or by a preferential increase in the amount of the specific DNA or RNA sequence, or by a
25 combination of the two. However, it should be noted that enriched does not imply that there are no other DNA or RNA sequences present, just that the relative amount of the sequence of interest has been significantly increased. The term "significant" is used to indicate that the level of increase is useful to the person making such an increase, and generally means an increase relative to other nucleic acids of about at least 2-fold, more preferably at least 5- to 10-fold or even more. The term also does not imply that there is
30 no DNA or RNA from other sources. The DNA from other sources may, for example,

comprise DNA from a yeast or bacterial genome, or a cloning vector such as pUC19. This term distinguishes from naturally occurring events, such as viral infection, or tumor-type growths, in which the level of one mRNA may be naturally increased relative to other species of mRNA. That is, the term is meant to cover only those situations in which a person has intervened to elevate the proportion of the desired nucleic acid.

Isolated DNA sequences are relatively more pure than in the natural environment (compared to the natural level this level should be at least 2- to 5-fold greater, *e.g.*, in terms of mg/mL). Individual sequences obtained from PCR may be purified to electrophoretic homogeneity. The DNA molecules obtained from this PCR reaction could be obtained from total DNA or from total RNA. These DNA sequences are not naturally occurring, but rather are preferably obtained via manipulation of a partially purified naturally occurring substance (*e.g.*, messenger RNA (mRNA)). For example, the construction of a cDNA library from mRNA involves the creation of a synthetic substance (cDNA) and pure individual cDNA clones can be isolated from the synthetic library by clonal selection from the cells carrying the cDNA library. The process which includes the construction of a cDNA library from mRNA and isolation of distinct cDNA clones yields an approximately 10^6 -fold purification of the native message. Thus, purification of at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated.

The term "gene encoding" refers to a sequence of nucleic acids which codes for a protein or polypeptide of interest. The nucleic acid sequence may be either a molecule of DNA or RNA. In preferred embodiments, the molecule is a DNA molecule. In other preferred embodiments, the molecule is a RNA molecule. When present as a RNA molecule, it will comprise sequences which direct the ribosomes of the host cell to start translation (*e.g.*, a start codon, ATG) and direct the ribosomes to end translation (*e.g.*, a stop codon). Between the start codon and stop codon is an open reading frame (ORF). One skilled in the art is very familiar with the meaning of these terms.

The term "insect cell lines" refers to cell lines derived from insects and susceptible to infection by the baculovirus. One skilled in the art is familiar with such cell lines and the techniques needed to utilize them. Representative examples of insect cell lines include *Spodoptera frugiperda* (sf9) and *Trichoplusia ni* (Hi-5) cell lines.

The terms "*Trichoplusia ni* (High-5) cells" and "*Spodoptera frugiperda* (sf9) cells" refers to insect cell lines used in combination with baculovirus expression vectors. One skilled in the art is familiar with these cell lines and how to obtain them.

The term "inserting" refers to a manipulation of a DNA sequence via the use of
5 restriction enzymes and ligases whereby the DNA sequence of interest, usually encoding the gene of interest, can be incorporated into another nucleic acid molecule by digesting both molecules with appropriate restriction enzymes in order to create compatible overlaps and then using a ligase to join the molecules together. One skilled in the art is very familiar with such manipulations and examples may be found in Sambrook *et al.*
10 (Sambrook, Fritsch, & Maniatis, "Molecular Cloning: A Laboratory Manual", 2nd ed., Cold Spring Harbor Laboratory, 1989), which is hereby incorporated by reference in its entirety including any drawings, figures and tables.

The term "adjuvant" refers to a substance which is provided with the antigen or immunogen of choice, *e.g.*, the protein or polypeptide to which an immune response is
15 desired, to enhance the immune response when one attempts to raise an immune response in an animal against the antigen or immunogen of choice. One skilled in the art is familiar with appropriate adjuvants to select and use. Adjuvants approved for human use include aluminum salts and MF59 (Singh and O'Hagan, *Nature Biotech* 17:1075-81, 1999). Other adjuvants are being developed (*Id.*) and may be used in conjunction with the present
20 invention.

The term "keyhole-limpet hemocyanin" or "KLH" refers to a protein which is isolated from keyhole limpets which is commonly used as a carrier protein in the immunization process. One skilled in the art is familiar with the meaning of the term keyhole limpet hemocyanin.

25 The term "cytokine" refers to a family of growth factors, soluble (glyco)proteins, secreted primarily from leukocytes. Cytokines stimulate both the humoral and cellular immune responses, as well as the activation of phagocytic cells. Cytokines are synthesized, stored and transported by various cell types not only inside of the immune system (lymphokines, interleukins, monokines, tumor necrosis factors, interferons) but
30 also by other cells which are associated with the study of hematology (colony-stimulating

factors), oncology (transforming growth factors), and cell biology (peptide growth factors, heat shock and other stress proteins).

Cytokines secreted from lymphocytes are termed lymphokines, while those secreted by monocytes or macrophages are referred to as monokines. Many of the lymphokines are also referred to as interleukins (ILs), since they are not only secreted by leukocytes but they are also able to affect the cellular responses of leukocytes. Specifically, interleukins are growth factors targeted to cells of hematopoietic origin.

The term "growth factor" refers to a protein that binds receptors on the surface of a cell and subsequently activates cellular proliferation and/or differentiation. Many growth factors are quite versatile and can act to stimulate cellular division in a wide variety of cell types, while others are specific to a particular cell-type.

The term "chemokine" refers to a group of small proinflammatory cytokines which function as chemoattractants and activators for leukocytes and represent a superfamily of over 30 chemotactic cytokines. They orchestrate the activation and migration of immune system cells from the blood or bone marrow to the site of infection and damaged tissue. Chemokines also play an essential role in the growth and proliferation of primitive stem cells found in bone marrow which in turn develop into mature immune cells. Chemokines are involved in a wide range of acute and inflammatory diseases and exert their action by binding to receptors of the seven-transmembrane-helix class.

Chemokines frequently range from 8 to 11 kDa in molecular weights, are active over a concentration range of 1 to 100 ng/ml, and are produced by a wide variety of cell types. The production of chemokines typically is induced by exogenous irritants and endogenous mediators such as IL-1, TNF-alpha, and PDGF. The chemokines bind to specific cell surface receptors and can be considered second-order cytokines that appear to be less pleiotropic than first-order proinflammatory cytokines because they are not potent inducers of other cytokines and exhibit more specialized functions in inflammation and repair.

The term "granulocyte-macrophage colony-stimulating factor" or "GM-CSF" refers to a small (less than 20 kDa) secreted protein. It binds to specific cell surface receptors and functions as species-specific stimulator of bone marrow cells. It stimulates the growth and differentiation of several hematopoietic cell lineages including dendritic

cells, granulocytes, macrophages, eosinophils, and erythrocytes. In particular, this cytokine also plays a role in shaping cellular immunity by augmenting T-cell proliferation (Santoli *et al.*, *J.Immunol.* 141(2):519-26, 1988), increasing expression of adhesion molecules on granulocytes and monocytes (Young *et al.*, *J.Immunol.* 145(2):607-15, 1990; 5 Grabstein *et al.*, *Science* 232(4749):506-08, 1986), and by augmenting antigen presentation (Morrissey *et al.*, *J.Immunol* 139(4):1113-9, 1986; Heufler *et al.*, *J. Exp. Med.* 167(2):700-05, 1988; Smith *et al.*, *J.Immunol.* 144(5):1777-82, 1990).

The term "monocyte chemotactic protein-3" or "MCP-3" refers a chemokine primarily produced by monocytes. MCP-3 has a wide spectrum of chemotactic activity 10 and attracts monocytes, dendritic cells, lymphocytes, natural killer cells, eosinophils, basophils, and neutrophils. The cDNA was cloned in 1993 by Minty *et al.*, *Eur Cytokine Netw* 4(2):99-110, 1993, and Opdenakker *et al.*, *Biochem Biophys Res Commun.*, 191(2):535-42, 1993. Its properties have been recently reviewed by Proost *et al.*, *J Leukoc Biol.* 59(1):67-74, 1996.

15 The term "expression vector" refers to a recombinant DNA construct which is designed to express a selected gene of interest, usually a protein, when properly inserted into the expression vector. One skilled in the art understands the term. Expression vectors commonly include a promotor at the 5' end of the site where the gene of interest is inserted and a terminator region at 3' end of the site. Frequently the gene of interest is inserted into 20 the appropriate site by means of selected restriction enzyme cleavage sites. The term "expression vector" also refers to a DNA construct such as described above into which the gene of interest encoding the product of interest has already been inserted.

The term "baculovirus expression vector" refers to a DNA construct which is designed to express a selected gene when used in the baculovirus system. Any of the 25 potential baculoviruses or expression vectors designed to function in the baculovirus system may be used in the instant invention. In a similar fashion, the term "expression vector" is a genus which encompasses the particular embodiment of baculovirus expression vectors, but "expression vectors" may function in cells and cell lines aside from, or in addition to, insect cell lines.

30 The term "allow the expression of" refers to placing an expression vector into an environment in which the gene of interest will be expressed. This commonly means

inserting the expression vector into an appropriate cell type where the promotor and other regions necessary for gene expression will be recognized by the host cell's components and will cause the expression of the gene of interest. The expression normally consists of two steps: transcription and translation. Expression can also be conducted in vitro using components derived from cells. One skilled in the art is familiar with these techniques, and such techniques are set forth in Sambrook *et al.* (Sambrook, Fritsch, & Maniatis, "Molecular Cloning: A Laboratory Manual", 2nd ed., Cold Spring Harbor Laboratory, 1989). In the preferred embodiment, the expressed product is a protein or polypeptide. In other preferred embodiments, the expressed product is $V_H/IgG_{\gamma 1}$, V_L/C_{κ} , V_L/C_{λ} , or $V_L/IgG_{\gamma 1}$.

The term "secretory signal sequence" refers to a peptide sequence. When this sequence is translated in frame as a peptide attached to the amino-terminal end of a polypeptide of choice, the secretory signal sequence will cause the secretion of the polypeptide of choice by interacting with the machinery of the host cell. As part of the secretory process, this secretory signal sequence will be cleaved off, leaving only the polypeptide of interest after it has been exported. In preferred embodiments, the honey bee melittin secretory signal sequence is employed. In other preferred embodiments, the human placental alkaline phosphatase secretory signal sequence is employed. The present invention is not limited by these secretory signal sequences and others well known to those skilled in the art may be substituted in place of, and in addition to, these. The term "secretory signal sequence" also refers to a nucleic acid sequence encoding the secretory peptide.

The term "ELISA" refers to "Enzyme-Linked ImmunoSorbent Assay" in which the presence or concentration of a protein is determined by its binding to the plastic well of an ELISA plate followed by its subsequent detection by antibodies specific for the protein to be quantified or detected.

The term "promoter controls" refers to an arrangement of DNA in an expression vector in which a promoter is placed 5' to a gene of interest and causes the transcription of the DNA sequence into an mRNA molecule. This mRNA molecule is then translated by the host cell's machinery. One skilled in the art is very familiar with the meaning of this term.

The terms "protein A," "protein G," and "protein L" refer to specific bacterial proteins which are capable of specifically binding immunoglobulin molecules without interacting with an antigen binding site. Protein A is a polypeptide isolated from *Staphylococcus aureus* that binds the Fc region of immunoglobulin molecules. Protein G is a bacterial cell wall protein with affinity for immunoglobulin G (IgG), which has been isolated from a human group G streptococcal strain (G148). Protein L is an immunoglobulin light chain-binding protein expressed by some strains of the anaerobic bacterial species *Peptostreptococcus magnus*.

The term "B cell lymphoma" refers to a cancer that arises in cells of the lymphatic system from B cells. B cells are white blood cells that develop from bone marrow and produce antibodies. They are also known as B lymphocytes.

The term "refractory low grade B cell lymphoma" refers to a low grade B cell lymphoma that has not responded to treatment. The term "low grade B cell lymphoma" refers to a lymphoma that tends to grow and spread slowly, including follicular small cleaved cell lymphoma. Also called indolent lymphomas due to their slow growth.

The term "follicular B cell lymphoma" refers to a type of non- Hodgkin's lymphoma. It is an indolent (slow-growing) type of lymphoma.

Further definitions and characterizations of low-grade lymphomas can be found on the Internet at http://rituxan.com/professional/clinical_information/class/index.html.

The term "isolating" as refers to a protein or polypeptide, refers to removing a naturally occurring polypeptide or protein from its normal cellular environment or refers to removing a polypeptide or protein synthesized in an expression system (such as the baculovirus system described herein) from the other components of the expression system. Thus, the polypeptide sequence may be in a cell-free solution or placed in a different cellular environment. The term does not imply that the polypeptide sequence is the only amino acid chain present, but that it is essentially free (about 90 - 95% pure at least) of non-amino acid-based material naturally associated with it.

By the use of the term "enriched" in reference to a polypeptide is meant that the specific amino acid sequence constitutes a significantly higher fraction (2- to 5-fold) of the total amino acid sequences present in the cells or solution of interest than in normal or diseased cells or in the cells from which the sequence was taken. This could be caused by

a person by preferential reduction in the amount of other amino acid sequences present, or by a preferential increase in the amount of the specific amino acid sequence of interest, or by a combination of the two. However, it should be noted that enriched does not imply that there are no other amino acid sequences present, just that the relative amount of the sequence of interest has been significantly increased. The term significant here is used to indicate that the level of increase is useful to the person making such an increase, and generally means an increase relative to other amino acid sequences of about at least 2-fold, more preferably at least 5- to 10-fold or even more. The term also does not imply that there is no amino acid sequence from other sources. The other source of amino acid sequences may, for example, comprise amino acid sequence encoded by a yeast or bacterial genome, or a cloning vector such as pUC19. In preferred embodiments, the amino acid sequence is a chimeric protein as described above. The term is meant to cover only those situations in which man has intervened to increase the proportion of the desired amino acid sequence.

It is also advantageous for some purposes that an amino acid sequence be in purified form. The term "purified" in reference to a polypeptide does not require absolute purity (such as a homogeneous preparation); instead, it represents an indication that the sequence is relatively purer than in the natural environment. Compared to the natural level this level should be at least 2-to 5-fold greater (*e.g.*, in terms of mg/mL). Purification of at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated. The substance is preferably free of contamination at a functionally significant level, for example 90%, 95%, or 99% pure.

The term "operatively linked" refers to an arrangement of DNA in which a controlling region, such as a promoter or enhancer, is attached to a connected DNA gene of interest so as to bring about its transcription, and hence allowing its translation. The term "operatively linked" may also refer to a DNA sequence encoding a processing signal, such as a secretory signal sequence, connected to a gene encoding a polypeptide to form a single open reading frame. Following transcription and translation, the secretory signal sequence has the potential to bring about the export of the translated polypeptide. One skilled in the art is familiar with the meaning of this term.

Functional Derivatives of Useful Chimeric Proteins

Also provided herein are functional derivatives of a polypeptide or nucleic acid of the invention. By "functional derivative" is meant a "chemical derivative," "fragment," or "variant," of the polypeptide or nucleic acid of the invention, as these terms are defined
5 below. A functional derivative retains at least a portion of the function of the protein, for example, reactivity with an antibody specific for the protein or binding activity mediated through noncatalytic domains, which permits its utility in accordance with the present invention. It is well known in the art that due to the degeneracy of the genetic code numerous different nucleic acid sequences can code for the same amino acid sequence.
10 Equally, it is also well known in the art that conservative changes in amino acid can be made to arrive at a protein or polypeptide that retains the functionality of the original. In both cases, all permutations are intended to be covered by this disclosure.

Included within the scope of this invention are the functional equivalents of the herein-described isolated nucleic acid molecules. The degeneracy of the genetic code
15 permits substitution of certain codons by other codons that specify the same amino acid and hence would give rise to the same protein. The nucleic acid sequence can vary substantially since, with the exception of methionine and tryptophan, the known amino acids can be coded for by more than one codon. Thus, portions or all of the genes of the invention could be synthesized to give a nucleic acid sequence significantly different from
20 a sequence that is found in nature. The encoded amino acid sequence thereof would, however, be preserved.

A "chemical derivative" of the complex contains additional chemical moieties not normally a part of the protein. Covalent modifications of the protein or peptides are included within the scope of this invention. Such modifications may be introduced into
25 the molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues, as described below. It may also consist of attaching carbohydrates to the protein in addition to the normal carbohydrates attached by the baculovirus expression system of the invention.

30 Cysteiny l residues most commonly are reacted with α -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give

carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylprocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing primary amine containing residues include imidoesters such as methyl picolinimide; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group.

Furthermore, these reagents may react with the groups of lysine as well as the arginine α -amino group.

Tyrosyl residues are well-known targets of modification for introduction of spectral labels by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimide ($R'-N-C-N-R'$) such as 1-cyclohexyl-3-(2-morpholinyl(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginy residues are frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

5 Other modifications include the in vitro glycosylation of polypeptides or proteins.

Derivatization with bifunctional agents is useful, for example, for cross-linking the component peptides of the protein to each other or to other proteins in a complex to a water-insoluble support matrix or to other macromolecular carriers. Commonly used cross-linking agents include, for example, 1,1-bis(diazoacetyl)-2-phenylethane,
10 glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[p-azidophenyl] dithiolpropioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence
15 of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include hydroxylation of proline and lysine, phosphorylation
20 of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains (Creighton, T.E., *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86, 1983), acetylation of the N-terminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.

25 Such derivatized moieties may improve the stability, solubility, absorption, biological half life, and the like. The moieties may alternatively eliminate or attenuate any undesirable side effect of the protein complex and the like. Moieties capable of mediating such effects are disclosed, for example, in *Remington's Pharmaceutical Sciences*, 18th ed., Mack Publishing Co., Easton, PA (1990).

30 A functional derivative of a protein with deleted, inserted and/or substituted amino acid residues may be prepared using standard techniques well-known to those of ordinary

skill in the art. For example, the modified components of the functional derivatives may be produced using site-directed mutagenesis techniques (as exemplified by Adelman *et al.*, *DNA* 2:183, 1983) wherein nucleotides in the DNA coding the sequence are modified such that a modified coding sequence is modified, and thereafter expressing this recombinant
5 DNA in a prokaryotic or eukaryotic host cell, using techniques such as those described above. Alternatively, proteins with amino acid deletions, insertions and/or substitutions may be conveniently prepared by direct chemical synthesis, using methods well-known in the art. The functional derivatives of the proteins typically exhibit the same qualitative biological activity as the native proteins.

10 **Uses of the Chimeric Proteins of the Invention**

Other aspects of the invention relate to uses for the instant chimeric proteins. Preferred uses include pharmaceutical and veterinary applications, wherein an effective amount of chimeric protein according to the invention (preferably in a composition according hereto) is administered to a patient. In this way, the chimeric protein contacts
15 cells of the patient, which contacting thereafter elicits the desired biological response. Methods for using the instant chimeric proteins include methods of eliciting an immune response in an organism, methods of raising antibodies (B cell immune response) in an organism, methods of inducing a T cell immune response by an organism, and methods for treating B cell pathologies. The invention also includes methods for treatment of subjects
20 in order to increase the immune response capable of altering a B cell pathology by administering a chimeric protein of the invention.

Typically, such methods are accomplished by delivering to the organism an effective amount of a chimeric protein according to the invention. "Effective amount" refers to an amount that results in the desired biological response being elicited. What
25 constitutes such an amount will vary, and depends on a variety of factors, including the particular chimeric protein, the desired biological response to be elicited, the formulation of the chimeric protein, the age, weight, gender, and health of the organism to be treated, the dosage regimen, the condition or disease to be treated or prevented, etc. Organisms to which the instant chimeric proteins and compositions may be administered include
30 mammals, preferably a mammal selected from the group consisting of a bovine, canine,

equine, feline, ovine, porcine, and primate animal. Particularly preferred organisms are humans.

The compounds described herein can be administered to a human patient *per se*, or in pharmaceutical compositions where it is mixed with other active ingredients, as in
5 combination therapy, or suitable carriers or excipient(s). Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition.

1. **Routes of Administration.**

Suitable routes of administration may, for example, include oral, rectal,
10 transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intravenous, intramedullary injections, as well as intrathecal, direct intraventricular, intraperitoneal, intranasal, or intraocular injections. One of skill in the art will understand the various modifications that would be made to adapt the composition to a particular route of administration.

15 Alternately, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into a solid tumor, often in a depot or sustained release formulation.

2. **Composition/Formulation.**

The pharmaceutical compositions of the present invention may be manufactured in
20 a manner that is itself known, *e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable
25 carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's
30 solution, or physiological saline buffer. For transmucosal administration, penetrants

appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art.

5 Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Suitable carriers include excipients such as, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth,
10 methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated
15 sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

20 Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds
25 may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

30 For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from

pressurized packs or a nebuliser, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch..

The compounds may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

A pharmaceutical carrier for the hydrophobic compounds of the invention is a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. The cosolvent system may be the VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:D5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system dissolves hydrophobic compounds well, and itself produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, *e.g.*, polyvinyl pyrrolidone; and other sugars or polysaccharides may substitute for dextrose.

Alternatively, other delivery systems for compositions may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Many of the compounds of the invention may be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be

formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms.

3. Effective Dosage.

5 Pharmaceutical compositions suitable for use in the present invention include compositions where the active ingredients are contained in an amount effective to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount of compound effective to prevent, alleviate or ameliorate symptoms of disease or
10 prolong the survival of the subject being treated. Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

 Toxicity and therapeutic efficacy of the compounds described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD₅₀ (the dose lethal to 50% of the population) and the
15 ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD₅₀ and ED₅₀. Compounds which exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in
20 formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See *e.g.*, Fingl *et al.*, "The Pharmacological Basis of Therapeutics,"
25 Ch. 1 p.1, 1975).

 Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the required effect, or minimal effective concentration (MEC). The MEC will vary for each compound. Dosages necessary to achieve the MEC will depend on individual characteristics and route of

administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals can also be determined using MEC value. Compounds should be administered using a regimen which maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%.

In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

4. Packaging.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accompanied with a notice associated with the container in form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the polynucleotide for human or veterinary administration. Such notice, for example, may be the labeling approved by the U.S. Food and Drug Administration for prescription drugs, or the approved product insert. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition. Suitable conditions indicated on the label may include treatment of a tumor, treatment of rheumatoid arthritis, treatment of diabetes, and the like.

EXAMPLES

In the following description, reference will be made to various methodologies known to those skilled in the art of immunology, cell biology, and molecular biology. Publications and other materials setting forth such known methodologies to which

reference is made are incorporated herein by reference in their entireties as though set forth in full.

5 1. **TISSUE PROCESSING FOR NON-HODGKIN'S LYMPHOMA IDIOTYPE
(ID) IDENTIFICATION AND CLONING:**

Tumor samples from a peripheral lymph node were biopsied as clinically indicated under sterile conditions and used to generate patient idiotype-specific recombinant chimeric immunoglobulin proteins. Remaining lymph node biopsy material was stored in liquid nitrogen in tissue cell bank for future use.

10 a. Cell Isolation: Single cell suspensions of patient lymph node biopsies were obtained by forcing the biopsied lymphoma tissue through a disposable 0.38 mm steel mesh screen while submerged in sterile PBS. The dispersed cells were washed twice in PBS, then resuspended and counted. A 10% fraction of the cells were processed for total RNA extraction and the remaining cells were archived in liquid nitrogen following
15 resuspension in RPMI 1640 tissue culture media containing 30% (v/v) fetal bovine serum and 10% (v/v) DMSO. All processing of clinical samples was performed in a biological safety cabinet.

 b. Total RNA Preparation: Total RNA from homogenized lymph node cells was isolated using RNeasy Kit (Qiagen) as per manufacturer's instruction. Total RNA was
20 quantitated by spectrophotometry.

 c. cDNA Synthesis: Approximately 2.0 µg total RNA was used as template for first strand cDNA synthesis using the SuperScript Preamplification System (GIBCO-BRL) according to manufacturer's recommendation. Oligo (dT) provided with the kit was used to prime the cDNA.

25 d. PCR Amplification of Genes Encoding Lymphoma Heavy and Light
Chains: Both heavy and light chains from the lymphoma-specific immunoglobulins were identified as follows. Aliquots of the single stranded lymphoma cDNA were combined with a series of V_H and V_L leader sequence-specific oligonucleotide sense primers representing all known V_H, V_K, and V_λ subfamilies as listed in Table 1, paired with IgM,
30 IgG, IgA, or Ig_κ and Ig_λ constant region specific antisense primers. These samples were then amplified by PCR and analyzed by agarose gel electrophoresis.

Parallel reactions were conducted using cDNA prepared from the patient's peripheral blood lymphocytes. A comparison of PCR products generated by each pair of primers derived from samples containing normal PBL or lymph node biopsy cDNA would lead to the identification of the candidate tumor specific V_H and V_K or V_L subfamily over-represented in the lymphoma, and the isotype of the heavy and light chains. Candidate tumor V region gene products were then excised, and their nucleic acid sequence was determined to assess clonality. For each patient, two independent analyses were performed from starting cellular fractions.

One microliter of the cDNA reaction (representing 5% of the total cDNA reaction volume) was amplified for 35 cycles in 50 μ l volume using the HotStarTaq Master Mix Kit (Qiagen). Cycling conditions: 95 °C 15 min, 65 °C 4 min, 72 °C 1 min, followed by 94 °C 1 min, 61 °C 30 sec, 72 °C 1 min for 34 cycles; and a final extension step at 72 °C for 7 min. A 10 μ l aliquot of each reaction is analyzed by electrophoresis on a 1% agarose gel with ethidium bromide.

TABLE 1. Primer Sequences Used for Amplification of Lymphoma Heavy and Light Chains

'(GA)' means either a G or an A, '(TC)' means either a T or a C.

	PRIMER NAME	PRIMER SEQUENCE (5' 3')
491	$V_{H1}L$	TCACCATGGACTGGACCTGGAG SEQ ID NO:38
492	$V_{H2}L.1$	ACCATGGACATACTTTGTTCCACGC SEQ ID NO:39
493	$V_{H2}L.2$	ACCATGGACACACTTTGCTCCACGC SEQ ID NO:40
494	$V_{H3}L.1$	ACCATGGAGTTTGGGCTGAGCTG SEQ ID NO:41
495	$V_{H3}L.2$	ACCATGGAAGTGGGGCTCCGCTG SEQ ID NO:42
496	$V_{H4}L$	AAGAACATGAAACACCTGTGGTTCTTC SEQ ID NO:43
497	$V_{H5}L$	ATCATGGGGTCAACCGCCATCCT SEQ ID NO:44
498	$V_{H6}L$	ACAATGTCTGTCTCCTTCCTCATC SEQ ID NO:45
516	$V_{K1}L$	ACATGAGGGTCCCCGCTCAGC SEQ ID NO:46

517	V _{K2} L	TCAGCTCCTGGGGCTGCTAATG SEQ ID NO:47
515	V _{K3} L	CTTCCTCCTGCTACTCTGGCTC SEQ ID NO:48
518	V _{K4} L	GCAGACCCAGGTCTTCATTTCTC SEQ ID NO:49
519	V _{K5} L	CCAGGTTACCTCCTCAGCTTC SEQ ID NO:50
520	V _{K6} L	GGTTTCTGCTGCTCTGGGTTCC SEQ ID NO:51
522	V _{λ1} L	TCACTG (TC) (GA) CAGGGTCCTGGGC SEQ ID NO:52
523	V _{λ2} L	ACTCAGG (GA) CACAGG (GA) TCCTGG SEQ ID NO:53
524	V _{λ3} L.1	TTGCTTACTGCACAGGATCCGTG SEQ ID NO:54
525	V _{λ3} L.2	CTTGCTCACTTTACAGGTTCTGTG SEQ ID NO:55
526	V _{λ3} L.3	CTCACTCTTTGCATAGGTTCTGTG SEQ ID NO:56
527	V _{λ3} L.4	TCAACCTCTACACAGGCTCTATTG SEQ ID NO:57
528	V _{λ3} L.5	CTCACTCTCTGCACAG (GT) CTCTG (AT) G SEQ ID NO:58
529	V _{λ4} .L1	CATTTTCTCCACAGGTCTCTGTGC SEQ ID NO:59
530	V _{λ4} L.2	CCTCCACTG (GC) ACAGGGTCTCTC SEQ ID NO:60
531	V _{λ5} L	CTCTCACTGCACAGGTTCCCTC SEQ ID NO:61
532	V _{λ6} L	CGCTCACTGCACAGGTTCTTGG SEQ ID NO:62
533	V _{λ7} L	CTTGCTGCCCAGGGTCCAATTC SEQ ID NO:63
534	V _{λ8} L	TGCTTATGGATCAGGAGTGGATTC SEQ ID NO:64
535	V _{λ9} L	CAGTCTCCTCACAGGGTCCCTC SEQ ID NO:65
536	V _{λ10} L	TCACTCACTCTGCAGTGTCAAGT SEQ ID NO:66
	IgG Constant- E	CTGAGTTCCACGACACCGTCAC SEQ ID NO:69
	IgM Constant- E	GGGAATTCTCACAGGAGACGAGG SEQ ID NO:70

	C _k -E	TTGGAGGGCGTTATCCACCTTC SEQ ID NO: 71
	C _λ -E	GAAGTCACTTATGAGACACACCAG SEQ ID NO: 72
	IgG Constant-I	GGAAGTAGTCCTTGACCAGGCAG SEQ ID NO: 73
	IgM Constant-I	GGGAAAAGGGTTGGGCCCCGATGCAC SEQ ID NO: 74
	C _k -I	GGGAAAAGGGTTGGGCCCCGATGCAC SEQ ID NO: 75
	C _λ -I	GGAACAGAGTGACACTGGGTGCAGCCTTGGGCTG SEQ ID NO: 76
	C _λ Downstream	TGCCGTCGGCAGGAGGTATTTTATTATGACTGTCT CCTTGCTATTATGAACATTCTGTAGGGGCCA SEQ ID NO: 77
	C _λ - 5'	GTCAGCCCAAGGCTGCACCCAGTGTCACTCTGTTCC SEQ ID NO: 78
	C _λ - 3'	CGTATCAAGCTTTTACTATGAACATTCTGTAGGGGC CAC SEQ ID NO: 79
	λ-stuff 1	CCTTTGATAACACCCA SEQ ID NO: 80
	λ-stuff 1'	GTGTTATCAAAGG SEQ ID NO: 81
	γ1-stuff 1	5'-CTAGTTTGATAAGGGCC-3' SEQ ID NO: 82
	γ1-stuff 1'	5'-CTTATCAAA-3' SEQ ID NO: 83
	κ-stuff 1	5'-CCTTTGATAACACCAA-3' SEQ ID NO: 84
	κ-stuff 1'	5'- -3' SEQ ID NO: 85

- e. Cloning and Sequencing of PCR Products: PCR products from reactions determined to contain the tumor specific variable sequences for heavy and light chains were cloned directly into plasmid pCR2.1-TOPO as per manufacturer's recommendations, and introduced into Top10 competent *E. coli* cells (Invitrogen). Twenty four miniprep DNA plasmids were prepared from carbenicillin resistant bacterial colonies using the QIAprep Spin Miniprep Kit (Qiagen), and quantitated by spectrophotometry. Two hundred ng of each plasmid was sequenced using the Cy5/Cy5.5 Dye Primer Cycle

Sequencing Kit (Visible Genetics). Following the completion of the sequencing reactions, samples were electrophoresed on the OpenGene Automated DNA Sequencing System and the data was processed with GeneObjects software package (Visible Genetics). Additional analysis including sequence alignments were performed using the SEQUENCHER

- 5 Version 4.1.2 DNA analysis software (GENE Codes Corp.). A V-region derived sequence could be considered tumor specific if it was present in 75% of the samples, for example, if 18 or greater of the 24 form a consensus group when analyzed using the above software utilizing the default parameters. Two independent biopsy samples would be compared when available.

- 10 f. cDNA Synthesis and Generation of 5' RACE Products: Due to the occurrence of mutations in the V_H and V_L sequences, it is not possible at times to identify tumor-specific immunoglobulin rearrangements. As an alternative to the sequence-specific PCR strategy *supra*, one can employ a 5' RACE PCR strategy to identify tumor specific immunoglobulin (Ig) rearrangements. All steps for first strand cDNA synthesis to
- 15 the generation of Ig specific PCR products are performed according to manufacturer's directions (5' RACE system for Rapid Amplification of cDNA Ends, version 2.0, Gibco BRL), with slight modification. Approximately 2.5 μ g of total RNA is used as template for each first strand cDNA synthesis in the presence of specific antisense primers complimentary to the immunoglobulin heavy and the light chains' constant region utilized
- 20 by the B cell population of interest (SEQ ID NO:69 for IgG, SEQ ID NO:70 for IgM, SEQ ID NO:71 for C_{κ} , and SEQ ID NO:72 for C_{λ}). The cDNA reactions are purified over GlassMAX spin cartridges, generating a final volume of 50 μ l each. A 10 μ l aliquot of each purified cDNA is oligo(dC) tailed with terminal deoxynucleotidyl transferase in a 25 μ l volume, generating the templates to be used for subsequent PCR reactions. The PCR
- 25 set up utilizes an upstream primer containing a poly(G) track provided by the manufacturer and an Ig specific antisense primer interior to that used for cDNA first strand synthesis (SEQ ID NO:73 for IgG, SEQ ID NO:74 for IgM, SEQ ID NO:75 for C_{κ} , and SEQ ID NO:76 for C_{λ}). Five μ l of template is amplified in a 50 μ l volume as follows: 95 $^{\circ}$ C for 15 min, 55 $^{\circ}$ C for 4 min, 72 $^{\circ}$ C for 1 min, followed by 94 $^{\circ}$ C for 1 min, 55 $^{\circ}$ C for
- 30 30 sec, 72 $^{\circ}$ C for 1 min for 34 cycles, and a final extension step at 72 $^{\circ}$ C for 7 min. The

final PCR products are separated by electrophoresis on a 1% agarose gel with ethidium bromide and the band of the appropriate size (~500-600 bp) is isolated and cloned into the pCR2.1-TOPO plasmid as described in 1e, *supra*.

5 2. **CONSTRUCTION OF BACULOVIRUS EXPRESSION VECTORS**
 pTRABacHuLC_KHC_{γ1} AND pTRABacHuLC_λHC_{γ1}

 a. Cloning of Secretory Signal Sequences into p2Bac: The base vector for the pTRABacHuLC_KHC_{γ1} and pTRABacHuLC_λHC_{γ1} constructs was p2Bac (Fig. 2, SEQ ID NO:5, Invitrogen, Carlsbad, CA). Two secretory signal sequences were cloned into this
10 base vector, and the first intermediate baculovirus expression vector p2BacM was created. In general, the vector p2Bac was first modified utilizing complimentary oligonucleotides encoding the amino terminal domain of the honey bee melittin secretory signal sequence positioned to be under transcriptional control of the baculoviral AcNPV P10 promoter. For melittin sequence cloning, 2 µg p2Bac was digested with Not I and Spe I for 4 hours
15 at 37 °C. The linear vector was purified following electrophoresis through a 1% agarose gel using Qiaex II resin (Qiagen, Chatsworth, CA). The purified DNA was then eluted with 50 µl water and the DNA concentration was determined. One µg each of primers Me1S/N (SEQ ID NO:15) and MeIN/S (SEQ ID NO:16) were mixed in 10 µl digestion
20 buffer M (Roche Molecular Biochemicals, Indianapolis, IN), and heated to 70 °C for 5 min, then cooled to room temperature to anneal complimentary primers. Ten percent of the annealed primers was digested in 20 µl reaction with Not I and Spe I for 4 hours at 37 °C, and the digested primers were purified following electrophoresis through a 15% polyacrylamide gel with Qiaex H resin, and the concentration of the DNA for annealed
25 primers was determined. The DNAs of p2Bac vector and annealed melittin fragment were ligated at 1:10 vector to insert ratio. The ligation product was transformed using competent XL1-Blue *E. coli* (Stratagene, San Diego, CA) and plated on a LB-carbenicillin agar plate for overnight growing at 37 °C. Miniprep colonies were prepared by standard protocols, and the plasmids were sequenced to check the construction. The resulting
30 vector p2BacM contained the melittin secretory signal sequence.

 The p2BacM vector was further modified similarly to encode for the amino terminal domain of the human placental alkaline phosphatase secretory signal sequence

under transcriptional control of the AcNPV polyhedron promoter, creating a second intermediate baculovirus expression vector p2BacMA. The procedure used to introduce the alkaline phosphatase sequence was generally cloned as follows: 2 µg p2BacM plasmid was digested with Bam HI and Eco RI, the linear vector was gel purified from agarose gel with Qiaex II resin and eluted in 50 µl water. The DNA concentration of the vector was determined. One µg each of primers APB/E (SEQ ID NO:17) and APE/B (SEQ ID NO:18) were mixed in 10 µl digestion buffer M, and heated to 70 °C for 5 min and then cooled down to room temperature to anneal complimentary primers. Ten percent of the annealed primers was digested in a 20 µl reaction with Bam HI and Eco RI for 4 hours at 37 °C. The digested primers were then purified from 15% polyacrylamide gel with Qiaex II resin. The DNA concentration of the digested primers was also determined. The linear p2BacM vector and alkaline phosphatase fragment were then ligated at 1:10 vector to insert ratio, and the ligation product was transformed using competent XL1-Blue *E. coli* and plated on a LB-carbenicillin agar plate for overnight growing at 37 °C. Miniprep colonies were prepared and the plasmids were sequenced to check the construction. The resulting intermediate vector p2BacMA would contain a secretory signal sequence for a human placental alkaline phosphatase. The p2BacMA plasmid was further transformed into SCS-110 *E. coli* strain (Stratagene) lacking *dcm* methylase activity for subsequent cloning of the κ constant region into methyl-sensitive Stu I site.

20 b. Amplification and Cloning of Constant Regions of IgG_{γ1} and Light Chains:

The human kappa (κ) constant and the human IgG_{γ1} constant domains of human monoclonal antibody 9F12 were PCR amplified from RNA extracted from the human cell line 9F12 (ATCC#HB8177). The κ constant region was cloned behind the alkaline phosphatase signal sequence. The IgG_{γ1} constant region was inserted downstream from the melittin secretory signal sequence thus creating the vector (pTRABacHuLC_κHC_{γ1}, Fig. 5a). A vector containing the human lambda (λ) light chain constant region (pTRABacHuLC_λHC_{γ1}, Fig. 5b) was produced by replacing the κ light chain constant region with a λ light chain constant region. The light chains were isolated by RT-PCR from a chronic lymphocytic leukemia cellular RNA preparation. The detailed description of the cloning procedures are as follows.

c. Amplification of 9F12 κ and IgG_{γ1} constant region fragments: Total RNA from 9F12 cells (ATCC#HB8177) was extracted using the RNeasy Kit (Qiagen) as per the manufacturer's instruction. A single stranded cDNA was synthesized using SuperScript reverse transcriptase (GIBCO BRL, Rockville, MD) with oligo(dT) primers. One
5 twentieth of the synthesized single strand cDNA was amplified in 100 μ l PCR reactions with Expand High Fidelity Taq (Roche) using κ and IgG_{γ1} specific oligonucleotides (SEQ ID NO:21 plus SEQ ID NO:22 and SEQ ID NO:19 plus SEQ ID NO:20, respectively). The fragments from amplified 9F12 immunoglobulin were purified from 1.5% SeaKem agarose with Qiaex II resin and eluted with 50 μ l water. The DNA concentrations for the
10 fragments were determined. The purified 9F12 immunoglobulin fragments were ligated separately into the TA-II (Invitrogen) PCR cloning vector. The ligation products were transformed using competent XLI-Blue *E. coli* and plated on a LB-carbenicillin agar plate for overnight growing at 37 °C. Miniprep colonies were prepared and the plasmid DNA was sequenced.

15 d. Insertion of the 9F12 κ Constant Region into the Expression Vector: For κ constant domain, 5 μ g plasmid DNA containing a κ constant region and 2 μ g of DNA for the vector p2BacMA purified from SCS110 *E. coli* were digested with Stu I and Hind III. A 320 bp fragment containing κ constant region and a 7.1 kb fragment containing p2BacMA vector were gel purified with Quiex II and eluted in 50 μ l water. The DNA
20 concentrations for both fragments were determined. The purified fragments were then ligated with Rapid Ligation Kit (Roche). The ligation products were transformed using competent XL1-Blue *E. coli* and plated on a LB-carbenicillin agar plate for overnight growing at 37 °C. Miniprep bacterial colonies were prepared and the recombinant DNA was sequenced to verify proper κ constant region insertion. The resulting plasmid vector
25 was pTRABacLC κ .

e. Addition of the IgG_{γ1} Constant Domain to the Vector: The IgG_{γ1} constant domain was added to the vector by first digesting 5 μ g of plasmid DNA containing IgG_{γ1} constant region and 2 μ g plasmid DNA for the vector pTRABacLC κ with Spe I and Xba I. A 1 kb fragment of IgG_{γ1} constant region and a 7.4 kb fragment of pTRABacLC κ vector
30 were gel purified from agarose plugs with Quiex II and eluted in 50 μ l water. The DNA

concentrations for both fragments were determined. The purified fragments were then ligated with Rapid Ligation Kit (Roche). The ligation products were transformed using competent XL1-Blue *E. coli* and plated on a LB-carbenicillin agar plate for overnight growing at 37 °C. Miniprep colonies were prepared and the ligation and orientation of the
5 IgG_{γ1} insertion were determined by restriction analysis and sequencing of the restriction sites. The resulting recombinant vector was pTRABacHuLC_κHC_{γ1}.

This plasmid, pTRABacHuLC_κHC_{γ1}, was further refined to add translational stop codons between the melittin secretory sequence, and the C_{γ1} region sequence and the alkaline phosphatase secretory sequence and the C_κ region sequence, respectively. To
10 accomplish these modifications, the pTRABacHuLC_κHC_{γ1} vector was linearized following digestion with Spe I + Apa I. The linearized vector was then ligated with annealed complimentary primers γ1-stuff 1 (SEQ ID NO:82) and γ1-stuff 1' (SEQ ID NO:83) to introduce the in-frame stop codons. The vector resulting from this modification was subsequently linearized following digestion with Stu I (AGGCCT) + Dra III
15 (CACnnnGTG) and then ligated with annealed complimentary primers κ-stuff 1 (SEQ ID No. 84) and κ-stuff 1' (SEQ ID NO:81) to introduce the in-frame stop codons. The net effect of these modifications are indicated in the sequences shown in Figures 6C & 6D, respectively. (The added sequences are highlighted by a double underline and bold.)

f. Addition of the λ Constant Region to the Vectors: Total RNA from
20 purified peripheral blood lymphocytes (PBL) obtained from a chronic lymphocytic leukemia (CLL) patient displaying a λ light chain idiotype was extracted using the RNeasy kit (Qiagen).

Approximately 2.0 μg total RNA was used as template for first strand cDNA synthesis using the SuperScript Preamplification System (Gibco BRL) according to
25 manufacturer's recommendation. Oligo(dT) was used for priming. One twentieth of the synthesized single stranded cDNA was amplified in a PCR reaction using an upstream primer identical to a portion of the Vλ signal sequence (SEQ ID NO:54) and a downstream primer (SEQ ID NO:77) complimentary to the last several codons of the λ constant region as well as a portion of the 3' untranslated region. The PCR products
30 were cloned into the pCRII vector (Invitrogen) and sequenced to confirm identity. A

plasmid containing the correct λ constant region sequence was chosen as a template for a second PCR. In this reaction a sense oligonucleotide, C λ -5' (SEQ ID NO:78), containing an engineered Dra III restriction site, corresponding the sequence in the λ constant region immediately downstream of J λ and a Hind III containing antisense oligonucleotide primer, C λ -3' (SEQ ID NO:79) spanning the STOP codon immediately following the λ constant region were utilized. The resulting PCR product was cloned into the pCR2.1-TOPO vector and sequenced. A fragment containing the λ constant region sequence was released upon Hind III restriction from some of the plasmids, depending on orientation of the insert. This restriction fragment was gel isolated and cloned into pTRABacHuLC κ HC γ 1 (Figure 5A), following linearization following Hind III digestion, generating an intermediate plasmid containing both the λ and κ constant regions. Restriction of this plasmid with Stu I and Dra III resulted in the removal of the κ sequences. This linearized plasmid was then ligated with annealed complimentary primers λ -stuff 1 (SEQ ID NO:80) and λ -stuff 1' to generate the final version of pTRABacHuLC λ HC γ 1 (Figure 5B).

15

3. INSERTION OF GENES FOR PATIENT-DERIVED IDIOTYPE V_H ND/OR V_L REGIONS INTO AN EXPRESSION VECTOR

Using either pTRABacHuLC κ HC γ 1 or pTRABacHuLC λ HC γ 1, it was possible to insert genes for any V_L region containing the unique cloning sequences Stu I and Dra III between the alkaline phosphatase signal sequence and the κ or λ constant region, and genes for any V_H region containing the unique cloning sequences *Spe* I and *Apa* I between the melittin secretory signal sequence and the IgG γ 1 constant region (See Figure 5A and 5B). The resulting expression vector could then be utilized for transduction into *Spodoptera frugiperda* (Sf-9) insect cells to produce recombinant budded baculovirus. The recombinant baculovirus was then serially amplified in Sf-9 cells to produce a high titer recombinant baculovirus stock. This high titer recombinant baculovirus stock was then used to infect *Trichoplusia ni* (High-5) cells for subsequent chimeric IgG protein production. A list of all oligonucleotide primers used in the construction of pTRABacHuLC κ HC γ 1 or pTRABacHuLC λ HC γ 1 can be found in Table 2.

After the tumor derived sequences for V_H and/or V_L regions are isolated as described above, oligonucleotide primers including the terminal 40 nucleotides of the melittin leader peptide (for Ig heavy chain cloning) (SEQ ID NO:8 – CAGATCACTA GTTTTATGG TCGTGTACAT TTCTTACATC TATGCG], the terminal 28 nucleotides
 5 of the alkaline phosphatase leader peptide (for Ig light chain cloning) (SEQ ID NO:9 – CTGAGTAGGC CTGAGGCTAC AGCTCTCCCT GGGC), and the first 21 to 24 nucleotides of the respective V_H or V_L region proteins are prepared. Reverse oligonucleotide primers from the heavy or light chain constant region are used (IgG: SEQ ID NO:10 – GGAAGTAGTC CTTGACCAGG CAG; IgM: SEQ ID NO:11 –
 10 GGGAAAAGGG TTGGGCCCGA TGCAC; Ig κ : SEQ ID NO:12 – GATGAAGACA CTTGGTGCAG CCACAG; Ig λ : SEQ ID NO:13: GGAACAGAGT GACACTGGGT GCAGCCTTGG GCTG). Recombinant plasmids identified previously as having the clonal V_H or V_L sequences are used as templates for a second round of PCR. Cycling conditions were as described *supra*.

15 Plasmid templates were combined with an IgG γ_1 , IgM, Ig λ , or Ig κ constant region primer complementary to codon encoding amino acids 141-149, 115-123, 108-119, and 109-117 respectively and the appropriate leader/V region fusion primer. For example, for one patient, the primers used were SEQ ID NO:67 for V_{H3} and SEQ ID NO:68 for $V_{\kappa 3}$ (SEQ ID NO:67: CAGATCACTA GTTTTATGG TCGTGTACAT TTCTTACATC
 20 TATGCGGAGA TGAAATTGGT GGAGTCTGGG; SEQ ID NO:68: CTGAGTAGGC CTGAGGCTAC AGCTCTCCCT GGGCGAAGTT GTGTTGACTC AGTCTCC). Cycling conditions were as described above.

a. Light Chain Variable Region Insertion into Expression Vector: A PCR derived V_L product and 2 μ g of the corresponding pTRABacHuLC κ HC γ_1 or
 25 pTRABacHuLC λ HC γ_1 cassette vector digested with *Stu* I and *Dra* III. The 350 bp DNA fragment from the patient derived V_L region and the 8.4 kb fragment for the linear pTRABacHuLC κ HC γ_1 or pTRABacHuLC λ HC γ_1 vector were purified from agarose gel plugs with Qiaex II resin and eluted in 50 μ l water. The DNA concentrations for both fragments were determined and then the fragments ligated using Rapid Ligation kit
 30 (Roche). The ligation products were used to transform competent XL1-Blue *E. coli* which

were subsequently plated on a LB-carbenicillin agar plate for overnight growing at 37 °C. Miniprep colonies were prepared and the recombinant DNA plasmids were verified by restriction analysis and sequencing. The resulting vector designated pTRABac(NHL-V_L)LC_κHC_{γ1} or pTRABac(NHL-V_L)LC_λHC_{γ1}.

- 5 b. Heavy Chain Variable Region Insertion into Expression Vector: A PCR derived V_H product and 2 μg of the pTRABac(NHL-V_L)LC_κHC_{γ1} or pTRABac(NHL-V_L)LC_λHC_{γ1} cassette vector were digested with *Spe* I and *Apa* I. The 350 bp DNA fragment from the patient derived V_H region and the 8.8 kb fragment for the linear pTRABac(NHL-V_L)LC_κHC_{γ1} or pTRABac(NHL-V_L)LC_λHC_{γ1} vector were purified from
10 agarose gel plugs with Qiaex II resin and eluted in 50 μl water. The DNA concentrations for both fragments were determined and then the fragments ligated using Rapid Ligation kit (Roche). The ligation products were used to transform competent XL1-Blue *E. coli* which were subsequently plated on a LB-carbenicillin agar plate for overnight growing at 37 °C. Miniprep colonies were prepared and the recombinant DNA plasmids were
15 verified by restriction analysis and sequencing. The resulting vector is designated pTRABac(NHL-V_L)LC_κ(NHL-V_H)HC_{γ1}, pTRABac(NHL-V_L)LC_λ(NHL-V_H)HC_{γ1} and is assigned a reference number corresponding to a patient, e.g., FV8786-001.

TABLE 2. Primer Sequences Used for Construction of pTRABacHuLC_κHC_{γ1} and pTRABacHuLC_λHC_{γ1} Baculovirus Transfer Vectors.

PRIMER NAME	PRIMER SEQUENCE (5' 3')
1. Melittin N-terminus (MeIS/N and MeIN/S)	ACTAGTGCAACGTTGACTAAGAATTTTCATGCGGCCGC (SEQ ID NO:15) GCGGCCGCATGAAATTCTTAGTCAACGTTGCACTAGT (SEQ ID NO:16)
2. Human Placental Alkaline Phosphatase N-terminus (APB/E and APE/B)	GCGGATCCATGGTGGGACCCTGCATGCTGCTGCTGCTGCTGCTAGGCCTggaattcc (SEQ ID NO:17) GGAATTCAGGCCTAGCAGCAGCAGCAGCAGCAGCAGCA TGCAGGGTCCCACCATGGATCCGC (SEQ ID NO:18)
3. IgG _{γ1} Heavy Chain Constant: Upstream	TGTGACTAGTATGTATCGGCCCATCGGTCTTCCCCCT (SEQ ID NO:19)
Downstream	TTTCTAGACTATTATTTACCCGGAGACAGGGAGAG (SEQ ID NO:20)

4. Kappa Light Chain Constant: Upstream	CTAGGCCTATGTATCACCAAGTGTCTTCATCTTCCCGC CATCT (SEQ ID NO:21)
Downstream	CCCAAGCTTCTATTAACACTCTCCCCTGTTGAAGCT (SEQ ID NO:22)

4. **TRANSFECTION OF INSECT CELL LINES WITH VARIABLE
REGION-CONTAINING EXPRESSION VECTORS AND PRODUCTION
OF RECOMBINANT CHIMERIC PROTEINS:**

- 5 a. **Insect Cell Growth:** Two established insect cell lines (*Sf9* and High-5) were transfected with modified baculoviral vectors to produce recombinant chimeric V_H /immunoglobulin and/or V_L /immunoglobulin proteins. All insect cells were grown at 28 °C in ESF-921 Serum Free Insect Media (Expression Systems LLP) containing 50 µg/L gentamycin in disposable sterile vented shaker flasks (Coming), at 140-150 rpm, with no
10 more than 50% liquid volume. Cells were passaged every 2 to 3 days. Frozen cells were thawed (Cryo-preservation media: 10% DMSO, 40% ESF-921 medium, 50% High-5 conditioned media) from a working cell bank for each lot of product or every six weeks to assure a continuous stock of exponentially growing cells that was not retractile to infection by baculovirus.
- 15 b. ***Sf9* Cell Transfection and Recombination Assay:** The modified baculovirus expression vectors containing genes for V_H and/or V_L regions and genes encoding immunoglobulin heavy and/or light chain constant regions were co-transfected into *Sf9* cells using the BacVector-3000 transfection kit (Invitrogen). Ten individual
20 plaques are picked from agarose overlays. Virus from isolated plaques are used to infect T-25 flasks seeded with *Sf9* cells at 50% confluency in 5 ml ESF-921 media. Clonal viral isolates amplified in T-25 flasks are tested by PCR, using two primers (SEQ ID NO:36 – TTTACTGTTT TCGTAACAGT TTTG) and (SEQ ID NO:37 – GGTCGTTAAC AATGGGGAAG CTG) to assure clonality of the isolated plaques and that there was no
25 wild type virus contamination. In general, 200 ng recombinant transfer vector plasmid was co-transfected with triple-cut Bac-Vector-3000 as described in the Bac Vector manual (Novagen) using the Eufectin lipid reagent supplied. This transfection mixture was subjected to serially 5-fold dilutions. One hundred microliter aliquots were plated in 60 mm tissue culture dishes containing 2.5×10^6 adherent *Sf9* cells. After 1 hour, cells were

overlaid with 4 ml of a 1% agarose solution in ESF-921 culture medium. Ten individual clones were picked from the transfected cells grown in agarose overlays after staining for live cells using Neutral Red (Sigma, St. Louis, MO) at $t=144$ hours post transfection.

Virus was eluted from plaque plugs overnight in 1 ml ESF-921 media. T-25 flasks were
5 seeded with Sf-9 cells at 50% confluency in 5 ml ESF-921 media, and infected with 0.5 ml of eluted clonal virus. Ninety-six hours post infection, 0.5 ml media was removed from T-25 flasks; the cells were removed by centrifugation and the supernatant was assayed for immunoglobulin activity by dot blotting on nitrocellulose. The absence of wild type virus was also tested by PCR as follows.

10 Infectious supernatant (10 μ l) containing recombinant baculovirus was added to 90 μ l of lysis buffer containing 10 mM Tris pH 8.3, 50 mM KCl, 0.1 mg/ml gelatin, 0.45% Nonidet P-40, and 0.45% Tween-20, containing 6 μ g Proteinase-K. The mixture was heated for 1 hour at 60 °C and the Proteinase-K was denatured by incubation at 95 °C for 10 min. Twenty five μ l of the heated mixture was removed to a fresh PCR tube after
15 cooling, and another 25 μ l of the mixture containing 10 mM Tris pH 8.3, 50 mM KCl, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween-20, 400 μ M each dNTP, 5 mM $MgCl_2$, 50 pM each PCR primer (final), and 2.5 U Taq polymerase (Roche) was added. The viral DNA was amplified for 40 cycles at: 92 °C for 1 min., followed by 58 °C for 1 min. and 72 °C for 1 min. The recombinant baculovirus primers PH forward (SEQ ID NO:36) and
20 PH reverse (SEQ ID NO:37) were used to amplify the polyhedron locus expressing the light-chain gene. PCR products were analyzed following electrophoresis through an agarose gel. Recombinant baculovirus would amplify a 1300 bp fragment, while wild type baculovirus would produce a ~ 800 bp fragment with these primer sets. Recombinant virus contaminated with wild type virus would amplify both fragment sizes.

25 **c. Preparation of High Titer Viral Stocks in Sf9 Insect Cells:** Two ml from a T-25 primary culture was transferred to a T-75 flask containing Sf9 cells at 50% confluency in 10 ml ESF-921 media, and cells were grown for 120 hours at 28 °C. Five ml of secondary T-75 cultures was transferred to a 150 ml shaker flask containing 50 ml of Sf9 cells at 2×10^6 cells/ml, and cells were grown for 120 hours at 28 °C. 25 ml was
30 transferred from the 150 ml shaker flask into 500 ml of Sf-9 cells at 2×10^6 cells/ml in a

one liter shaker flask, and was grown at 28 °C. When the cultures reached 20%, viable cells as determined by trypan blue staining (approximately 120 to 144 hours post infection), the viral culture was harvested by centrifugation at 3000 x g, distributed into 50 ml sterile tubes, and half of the tubes were stored at 4 °C with the rest at -80 °C. This
5 harvested 500 ml high titer ($>1 \times 10^8$ pfu/ml) viral stock was then used to infect High-5 insect cells for immunoglobulin production. Viral titers (pfu/ml) were determined using a Baculovirus Rapid Titer Kit (Clontech, Palo Alto, CA).

d. **Production of Id in High-5 Insect Cells:** High-5 insect cells (BTI-TN-5B1-4) secreted higher levels (2-20 X) of recombinant immunoglobulin compared to Sf9
10 cells, and were chosen for chimeric protein production. Early log phase High-5 cells ($1.0-2.0 \times 10^6$ cells/ml) were seeded in 1 liter disposable culture flasks with vented closures at 5×10^5 cells/ml in ESF921 Media (Expression Systems LLP). The flasks were shaken at 140-150 rpm at 28 °C, and the volume of media in the flasks was adjusted over time to no greater than 500 ml. When the cell densities reached 1.5 - 2.5 cell/ml in 500 ml media, the
15 flasks were infected with high titer recombinant baculovirus stock at a multiplicity of infection (MOI) approximating 0.5:1 (pfu:cells). The flasks were then shaken at 140-150 rpm at 28 °C; the culture was harvested 96 hours post-infection.

20 5. **PURIFICATION OF THE CHIMERIC PROTEIN COMPRISING A V_H-IMMUNOGLOBULIN AND A V_L-IMMUNOGLOBULIN:**

Cells and debris were removed by centrifugation for 60 min. at approximately 5,000 x g, followed by filtration through a 0.2µ PES sterile filter unit. Chimeric proteins were purified from cleared tissue culture media by affinity chromatography with a Protein-A High-Trap cartridge (Amersham Pharmacia, Piscataway, NJ), followed by ion-exchange
25 chromatography utilizing FPLC technology (Amersham Pharmacia). The purified chimeric proteins were size separated and buffer exchanged into PBS by FPLC chromatography. All reagents used for protein purification were of USP biotechnology grade (GenAr, Mallinckrot Baker, Parris, KY) and endotoxin tested by the manufacturer. Sterile USP grade water was used to make all buffers and other solutions. Buffers and
30 other solutions were prepared in a biological safety cabinet, and filter sterilized through 0.2 µm PES filter units.

a. **Protein A Sepharose Affinity Purification of the Chimeric Proteins:**

Tissue culture medium was removed from growing culture flasks and spun for 60 min. at 5,000 x g to sediment cells and debris. The supernatant was sterilized by filtration using a 0.2 μ PES filter unit. Tris buffer (1M, pH 7.4) was added to the filtered medium
5 containing V_H and/or V_L-immunoglobulin chimeric proteins to a final concentration of 20 mM. The buffered tissue culture supernatant was loaded onto a 5 ml HighTrap recombinant Protein A Sepharose affinity cartridge at a flow rate of 1 to 5 ml/min with a P1 peristaltic pump (Amersham Pharmacia) collecting the flow-through in a clean flask. The column was washed with 25 ml PBS (pH 7.4) at 5 ml/min. The direction of the flow
10 was reversed and the column was washed with an additional 25 ml PBS. The column was eluted in reverse at 1 ml/min with 0.05 M citric acid (pH 3.5) collecting 1 ml fractions. Other protein columns including but not limited to protein G, protein L, or any proteins that are able to bind to an immunoglobulin binding domain could be used in the same manner.

15 b. **Ion Exchange Chromatography:** A 5 ml High Trap SP Sepharose cation exchange cartridge was equilibrated with 50 ml of 25 mM citric acid (pH 3.5) and 20 mM NaCl. The Protein A eluted V_H and/or V_L-IgG chimeric proteins were loaded directly onto the equilibrated High Trap SP Sepharose column using a peristaltic pump at a flow rate of 1 ml/min. The column was washed with 25 ml 50 mM citric acid (pH 3.5) and 20 mM
20 NaCl (Buffer A) at 2 ml/min. The column was eluted with a linear gradient (0% Buffer B to 100% Buffer B) to collect 1 ml fractions at 1 ml/min. (Buffer B = 100 mM Na carbonate (pH 10.0) and 1M NaCl). The ion exchange eluted fractions containing V_H and/or V_L-IgG chimeric proteins were analyzed spectrophotometrically by their OD₂₈₀. The peak fractions were pooled.

25 c. **Size Exclusion Chromatography:** The pooled Ig fraction from SP ion-exchange was then loaded onto a Hi Prep Sephacryl 26/60 S200 Hi Resolution column (Pharmacia) that had been equilibrated in 5 column volumes of PBS (pH7.2) following a pre-wash in 100 ml sterile water. The chimeric Ig proteins were eluted in PBS at a flow rate of 0.5 ml/min and collected in 1 ml fractions. The major Ig peak was apooled a sterile
30 filtered through a 0.2 μ filter.

6. **IDIOTYPIC PROTEIN AND KEYHOLE LIMPET HEMOCYANIN (KLH) CONJUGATION.**

Once purified, the idiotypic protein was conjugated to GMP grade KLH (VACMUNE, Biosyn Corporation) via glutaraldehyde crosslinking. At least 5 mg of purified, sterile idiotypic protein as described, *supra*, was combined with an equal weight of KLH in a sterile 15 ml conical tube and the final volume was adjusted to 9 ml in PBS. One ml of 1% glutaraldehyde (25% Grade 1 aqueous solution, Sigma) was added dropwise to a final concentration of 0.1%. The tube was then slowly rocked for 4 hours at room temperature. The conjugate was dialyzed in sterile DispoDialyzers (Spectrum Labs) against 2 liters sterile PBS, with three buffer changes over at least 24 hours in a biological safety hood. The final IgG/KLH conjugate in PBS is aseptically removed from the dialysis chambers and transferred into a sterile tube, mixed, then aliquoted in vials. Each vial of final product was labeled with the lot number, patient identifier, vial number and date vialled. Ten percent of the final vialled lot was tested for sterility and a vial was tested for the presence of endotoxin. One vial was retained for archival purposes.

7. **PRODUCT TESTS**

a. **DNA Sequence of Baculovirus Containing Production Lot**

Supernatant: A 1 ml aliquot of sample of infected insect cell production culture supernatant was harvested and cleared of cellular debris by spinning at 3000 rpm for 5 min in a desktop centrifuge. At least 0.1 ml of this cleared supernatant containing baculovirus particles was combined at a volume ratio of 1 to 9 with lysis buffer (10mM Tris pH 8.3, 50 mM KCl, 0.1 mg/ml gelatin, 0.45% Nonidet P-40, and 0.45% Tween-20), subjected to proteolysis with proteinase K (final concentration 60 µg/ml) for 1 h at 60 °C, followed by denaturation for 15 min at 95 °C. Twenty-five µl of this lysate was then combined with an additional 25 µl of the above lysis buffer containing 400 µM each dNTP, 5 mM MgCl₂, 25 pmol forward and reverse oligonucleotide primers (see Table 3; SEQ ID NO:34 and SEQ ID NO:31 for V_H Identification and SEQ ID NO:35 and SEQ ID NO:36 for V_L identification, respectively), and 2.5 U Taq polymerase (Roche). Cycling conditions for the PCR of V_L are: initial denaturation for 2 min at 92 °C, followed by 40 cycles of 1 min each at 92 °C, 58 °C, and 72 °C, with a final extension of 7 min at 72 °C. For the PCR of

V_H, cycling conditions are the same except that the annealing temperature is 64 °C. PCR products were assessed for expected size and quantity by agarose gel electrophoresis. Subsequently, two or more nested primers were used to directly sequence the PCR products. (See Table 3; SEQ ID NO:30 and SEQ ID NO:34 for V_H identification, SEQ ID NO:28 and SEQ ID NO:35 for V_K identification, and SEQ ID NO:88 and SEQ ID NO:35 for V_λ identification, respectively.) The complete V_H and V_L nucleotide sequences was determined using the the OpenGene Automated DNA Sequencing System (Visible Genetics) and sequencing analysis software, as described above and compared with the V-gene sequences of the pTRABac(NHL-FV-8786-XXX) vector corresponding to that patient's idiotype.

b. Superose 6 Gel Filtration Chromatography: Gel filtration chromatography of the purified Id was performed to assess protein purity. Gel filtration chromatography was performed using a Superose 6 HR 10/30 FPLC column (Amersham Pharmacia) with PBS as the liquid phase. Peak integration was performed on the largest 20 peaks by the FPLC software using the following criteria to reject a peak from being included in area evaluation: height less than 0.01 Au; width less than 0.05 ml; area less than 0.01 Au/ml. Fractions of each column run were collected and assayed for human immunoglobulin specific activity by capture ELISA, and compared to the OD₂₈₀ chromatogram.

c. Immunoglobulin Assay; Anti Human IgG ELISA: Microtiter plate wells were coated with 100μl of a 3 μg/ml dilution of Goat anti-Human IgG heavy chain specific antibody (Roche) in carbonate buffer overnight at 4 °C, and washed 2 times with 100μl TBS (50mM Tris, 150mM NaCl, pH 7.5). Wells were blocked with of 200μl TBSB (TBS + 1% BSA) for 1 hour at 22 °C.

Each chromatogram fraction corresponding to human peak in TBSB was tested. One hundred μl of diluted sample was added in 2-fold serial dilutions to wells in replicates, and incubated 1 hour at 22 °C. The assay was repeated with purified Human IgG1/κ or IgG1/λ standards (Sigma, St. Louis, MO). The wells were washed 4 times with 200μl TBST (TBS + 0.1% Tween 20). The detection antibody was diluted (Goat-anti-Human κ or λ-HRP (Fischer, Pittsburgh, PA)1:2000 in TBSB, 100μl was added to wells,

and incubated for 1 hour at 22 °C. The wells were washed 6 times with 200µl TBST. One hundred µl of substrate (TMB 1 component, KPL Inc., Gaithersburg, MD) was added to wells, developed 30 min. and assayed at OD₆₂₀.

- d. **Idiotypic Protein Release Criteria:** (1) The DNA sequence of idiotype-variable genes in baculovirus from production supernatant must be identical to the DNA sequence in the production vector. (2) The idiotypic protein concentration was greater than 0.5 mg/ml based on OD₂₈₀. (3) The major peak area was greater than 90% of area in evaluated peaks on Superose 6 analytical chromatography. (4) The major chromatographic peak corresponds to the human IgGκ (or λ) ELISA activity peak.

- 10 The final vaccine product, Id-KLH, was tested for endotoxin levels by a kinetic turbidity microplate assay or a Limulus Amoebocyte Lysate (LAL) assay and had a level below 350 endotoxin units (EU) per ml. Ten percent of the lot was tested for sterility on a 14-day test and tests negative or was discarded.

- 15 Table 3 shows a summary of primer sequences used for establishing final product identity.

TABLE 3. Primer Sequences Used for Establishing Final Product Identity.

PRIMER NAME	PRIMER SEQUENCE (5' 3')
1. Human Placental Alkaline Phosphatase Internal	AAATGATAACCATCTCGC (SEQ ID NO:25)
2. Human Placental Alkaline Phosphatase External	TTTACTGTTTTTCGTAACAGTTTTG (SEQ ID NO:26)
3. Kappa Light Chain Constant Antisense	TTGGAGGGCGTTATCCACCTTC (SEQ ID NO:27)
4. Kappa Light Chain Constant Downstream Internal	CTGTAAATCAACAACGCACAG (SEQ ID NO:28)
5. Kappa Light Chain Constant Downstream External	CAACAACGCACAGAATCTAG (SEQ ID NO:29)
6. Melittin Internal	GGGACCTTTAATTCAACCCAACAC (SEQ ID NO:30)
7. Melittin External	AAACGCGTTGGAGTCTTGTGTGC (SEQ ID NO:31)
8. IgG _{γ1} Heavy Chain Constant Downstream Internal	GGAAGTAGTCCTTGACCAGGCAG (SEQ ID NO:32)
9. IgG _{γ1} Heavy Chain Constant	CTGAGTTCCACGACACCGTCAC

Downstream Middle	(SEQ ID NO:33)
10. IgG _{γ1} Heavy Chain Constant Downstream External	TAGAGTCCTGAGGACTGTAGGAC (SEQ ID NO:34)

11. Kappa & Lambda Downstream:	5'-GGTCGTTAACAATGGGGAAGCTG-3' (SEQ ID NO:35)
12. PH forward	5'-TTTACTGTTTTTCGTAACAGTTTTG-3' (SEQ ID NO:36)
13. PH reverse	5'-GGTCGTTAACAATGGGGAAGCTG-3' (SEQ ID NO:37)
14. Lambda Constant Internal	5'-GAAGTCACTTATGAGACACACCAG-3' (SEQ ID NO:88)

8. **USE OF CHIMERIC PROTEIN OF THE INVENTION FOR
TREATMENT OF NON-HODGKIN'S B-CELL LYMPHOMA:**

- 5 V_H and V_L regions were obtained from a patient with Non-Hodgkin's B-Cell Lymphoma. Using the 5' RACE method described *supra*, genes encoding these regions were cloned and inserted into the expression vector and expressed by the methods of the instant invention. Table 5 contains the DNA sequences of the V_H and V_L regions used for the expression vector. The Apa I and Dra III sites used for cloning are indicated by
- 10 underlining.

Table 5: Variable region sequences obtained from a patient.

VH A / 07

GACATGTTGTTGGTGAATCGGGGGAGGCCTGGTCCAGCCGGGGAGTCCCTGAGACT
CTCCTGTGTGGCCTCTAGATTACCTTTAGAACGTTTTGGATGACCTGGGTCCGCCAAC
TTCCAGGGAAGGGGCTGGAGTGGGTGGCCAATATAAATCAAGATGGCAGTCAGACGTAT
CATGCGGACTCTGTAAAGGGCCGATTTACCATCTCCAGAGACAACGGCAGGAAGTCCCT
ATTTTTACAAATGACAAGTCTGAGAGTCGCGGACACGGCTATATATTACTGTGCGACTA
ATGAAACGTCCAGTGGCCTGGACTGCTGGGGCCAAGGAACCTGGTCACTGTCTCCTCA
GCTTCCACCAAGGGCCC
SEQ ID NO:86

VK A / L6

GAAATCGTGTTGACACAGTCTCCAGCCACCCTGTCTTCGTCTCCAGGAGACAGAGTCGC
CCTCTCTGCAGGGCCAGTCAGAGTGTAAAGAGTTACTTAAGTTGGTATCAACAGAAGG
CTGGCCAGGCTCCCAGGCTCCTCATCCATAATGCATCCAGTAGGGCCACTGGCATCCCG
CCCAGATTCAAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGTCGCCTAGA
GACTGAAGATGCTGCAGTTTATTACTGTGAGCAACTTTATTTCTGGCCTCCGATATTAT
TTTTCGGCCCTGGGACCAAGTGAATATCACACGAAGTGTGGCTGCACCAAGTG
SEQ ID NO:87

The isolated recombinant chimeric immunoglobulin protein produced for this patient from the genetic information detailed above was conjugated to KLH and administered with GM-CSF five times over a six-month period as described *supra*. A CT scan was performed on the neck and pelvis areas of the patient prior to administration of the therapy and 9 months later. A comparison of the sum of the diameters of 6 tumor masses revealed a 60% reduction nine months following therapy initiation. (Note that these figures are not adjusted to accommodate the size of the lymph node prior to diagnosis of the disease (See, Cheson et al., *J. Clin. Oncol.*, 17(4):1244, 1999.)

10 Table 6: Reduction in size of lymph nodes following treatment.

	PRIOR TO TXT. (Product of diameters; cm ²)	9 MONTHS POST TXT. (Product of diameters; cm ²)
LYMPH NODE 1	6.16	2.8
LYMPH NODE 2	5.0	1.6
LYMPH NODE 3	3.3	1.17
LYMPH NODE 4	3.78	1.44
LYMPH NODE 5	1.92	1.0
LYMPH NODE 6	1.08	0.80
SUM OF DIAMETERS	21.24	8.81

CLAIMS

1. A method for altering a B cell mediated pathology in a patient, said method comprising:
- administering a composition comprising a chimeric protein;
- 5 said chimeric protein comprising at least a portion of a V_H or V_L region and at least a portion of an immunoglobulin constant region;
- wherein said V_H or V_L region is associated with a B cell clone from said patient having said B cell mediated pathology;
- and said administering of said composition alters said B cell mediated pathology
- 10 in said patient.
2. The method of claim 1 wherein said composition further comprises a second chimeric protein that comprises at least a portion of V_H or V_L region and at least a portion of a second immunoglobulin constant region.
3. The method of claim 1 wherein said immunoglobulin constant region
- 15 comprises a human $IgG_{\gamma 1}$ constant region.
4. The method of claim 1 wherein said V_H or V_L region is a V_H region.
5. The method of claim 1 wherein said V_H or V_L region is a V_L region.
6. The method of claim 1 or 2 wherein said V_H or V_L region of said first chimeric protein comprises a V_H region and said second chimeric protein comprises a V_L
- 20 region.
7. The method of claim 2 wherein said second immunoglobulin constant region comprises a human κ or λ constant region.
8. The method of claim 1 or 2 wherein said V_H or V_L region is an entire variable region.

9. The method of claim 1 or 2 wherein said V_L region is an entire variable region.

10. The method of claim 1 or 2 wherein said first or second immunoglobulin constant region is selected from the group consisting of a human $IgG_{\gamma 1}$ constant region, a
5 human $IgG_{\gamma 2}$ constant region, a human $IgG_{\gamma 3}$ constant region, a human $IgG_{\gamma 4}$ constant region, a human IgA_1 constant region, a human IgA_2 constant region, a human IgM constant region, a human IgD constant region, a human IgE constant region, a human κ chain constant region, and a human λ chain constant region.

11. The method of claim 1 wherein said chimeric protein is produced by a
10 method comprising:

isolating a gene encoding at least a portion of a V_H or V_L region from B cells of said patient having said B cell mediated pathology;

inserting said gene encoding said V_H or V_L region and a gene encoding at least a portion of said immunoglobulin constant region into an expression vector to allow the
15 expression of said first chimeric protein;

producing said chimeric protein by introducing said expression vector into insect cell lines; and

isolating said chimeric protein.

12. The method of claim 11 further comprising the step of inserting a gene
20 encoding at least a portion of a V_H or V_L region and a gene encoding at least a portion of a second immunoglobulin constant region into said expression vector to allow the expression of said second chimeric protein.

13. The method of claim 11 or 12 further comprises a step of conjugating said chimeric protein to a carrier protein.

14. The method of claim 13 wherein said carrier protein is keyhole-limpet
25 hemocyanin (KLH).

15. The method of claim 1 wherein said composition is further co-administered with a cytokine or chemokine.

16. The method of claim 15 wherein said cytokine is granulocyte-macrophage-colony stimulating factor (GM-CSF).

5 17. The method of claim 15 wherein said chemokine is monocyte chemotactic protein 3 (MCP 3).

18. The method of claim 11 wherein said expression vector is a baculovirus expression vector.

10 19. The method of claim 18 wherein said baculovirus expression vector comprises a honey bee melittin secretory signal sequence and a human placental alkaline phosphatase secretory signal sequence.

20. The method of claim 19 wherein said baculovirus expression vector further comprises a baculovirus AcNPV p10 promotor and AcNPV polyhedrin promotor, wherein said p10 promotor controls a honey bee melittin secretory signal sequence, and wherein
15 said polyhedrin promotor controls a human placental alkaline phosphatase secretory signal sequence.

21. The method of claim 20 wherein a gene encoding a chimeric protein comprising a V_H region and a first immunoglobulin constant region is controlled by said p10 promotor in said baculovirus expression vector, a gene encoding a chimeric protein
20 comprising a V_L region and a second first immunoglobulin constant region is controlled by polyhedrin promotor in said baculovirus expression vector.

22. The method of claim 20 wherein said gene comprising said V_H or V_L region and said gene encoding said immunoglobulin constant region is controlled by either said p10 promotor or polyhedrin promotor in said baculovirus expression vector.

23. The method of claim 11 wherein said gene encoding said immunoglobulin constant region is a human IgG_{γ1} gene.

24. The method of claim 12 wherein said gene encoding said second immunoglobulin constant region is a gene encoding for a human κ or λ constant region.

5 25. The method of claim 11 or 12 wherein said gene encoding said immunoglobulin constant region is selected from the group consisting of a human IgG_{γ1} constant region, a human IgG_{γ2} constant region, a human IgG_{γ3} constant region, a human IgG_{γ4} constant region, a human IgA₁ constant region, a IgA₂ human constant region, a human IgM constant region, a human IgD constant region, a human IgE constant region, a
10 human κ chain constant region, and a human λ chain constant region.

26. The method of claim 11 wherein said chimeric protein is selected from the group consisting of a protein comprising said V_H region and a human IgG_{γ1} constant region; a protein comprising said V_L region and a human κ chain constant region; and a protein comprising said V_L region and a human λ chain constant region.

15 27. The method of claim 12 wherein said first and second chimeric proteins comprise a protein comprising said V_H region and a human IgG_{γ1} constant region and a protein comprising said V_L region and a human κ or λ chain constant region.

28. The method of claim 11 wherein said insect cell lines are *Trichoplusia ni* (Hi - 5) or *Spodoptera frugiperda* (sf9) cell lines.

20 29. The method of claim 11 or 12 wherein said chimeric proteins are analyzed for expression by ELISA.

30. The method of claim 11 or 12 wherein said chimeric proteins are isolated using a protein selected from the group consisting of protein A, protein G, protein L and other proteins being able to bind to an immunoglobulin binding domain.

31. The method of claim 30 wherein said other protein able to bind an immunoglobulin binding domain is an anti-immunoglobulin antibody.

32. The method of claim 1 wherein said B cell mediated pathology is a B cell lymphoma.

5 33. The method of claim 32 wherein said B cell lymphoma is refractory low grade lymphoma or follicular B cell lymphoma.

34. A composition for altering a B cell mediated pathology in a patient comprising:

10 a chimeric protein that comprises at least a portion of a V_H or V_L region linked to at least a portion of an immunoglobulin constant region, wherein said V_H or V_L region is associated with a B cell clone from said patient having said B cell mediated pathology.

35. A composition of claim 34 further comprising a second chimeric protein that comprises at least a portion of a variable region of a V_H or V_L region and at least a portion of a second immunoglobulin constant region, wherein said variable region is
15 associated with a B cell clone from said patient having said B cell mediated pathology.

36. The composition of claim 34 or 35 wherein said chimeric proteins are produced in according to claim 13 or 14.

37. The composition of claim 34 or 35 wherein said immunoglobulin constant regions are selected from the group consisting of a human $IgG_{\gamma 1}$ constant region, a human
20 $IgG_{\gamma 2}$ constant region, a human $IgG_{\gamma 3}$ constant region, a human $IgG_{\gamma 4}$ constant region, a human IgA_1 constant region, a human IgA_2 constant region, a human IgM constant region, a human IgD constant region, a human IgE constant region, a human κ chain constant region, and a human λ chain constant region.

38. The composition of claim 34 wherein said immunoglobulin constant region is a human IgG_{γ1} constant region operatively linked to said V_H region.

39. The composition of claim 34 wherein said immunoglobulin constant region is a human κ or λ constant region operatively linked to said V_L region.

5 40. The composition claim 35 wherein said first and second chimeric proteins are said V_H region operatively linked to a human IgG_{γ1} constant region and said V_L region operatively linked to a human κ or λ constant region.

41. The composition of claim 34 or 35 further comprises a carrier protein.

42. The composition of claim 41 wherein said carrier protein is keyhole-limpet
10 hemocyanin (KLH).

43. The composition of claim 34 or 35 is further co-administered with a cytokine or chemokine.

44. The composition of claim 43 wherein said cytokine is granulocyte-macrophage-CSF.

15 45. The composition of claim 43 wherein said chemokine is a monocyte chemotactic protein 3 (MCP 3).

46. The composition of claim 34 or 35 wherein said composition is a vaccine.

47. The composition of claim 34 wherein said B cell mediated pathology is a B cell lymphoma.

20 48. The composition of claim 47 wherein said B cell lymphoma is non-Hodgkins lymphoma.

49. The composition of claim 47 wherein said B cell lymphoma is refractory low grade or follicular B cell lymphoma.

50. The composition of claim 34 is further administered by injection, inhalation, oral or transdermal delivery.

5 51. The composition of claim 34 wherein said B cell mediated pathology is an autoimmune disease selected from the group consisting of multiple sclerosis, systemic lupus erythematosus, anti-Hu associated paraneoplastic neurological syndrome, autoimmune hepatitis, Type I diabetes, autoimmune thyroiditis, and scleroderma.

10 52. An expression vector comprising (a) a chimeric gene encoding a portion of a V_L region and a portion of a gene encoding a κ light chain, both operatively linked to a human placental alkaline phosphatase secretory signal sequence and a polyhedrin promoter, and (b) a chimeric gene encoding a portion of a V_H region and a portion of an IgG₁ heavy chain, both operatively linked to a honey bee melittin secretory signal sequence and a P10 promoter.

15 53. A baculovirus expression vector comprising (a) a chimeric gene encoding a portion of a V_L region and a portion of a gene encoding a κ light chain, both operatively linked to a human placental alkaline phosphatase secretory signal sequence and a polyhedrin promoter, and (b) a chimeric gene encoding a portion of a V_H region and a portion of an IgG₁ heavy chain, both operatively linked to a honey bee melittin secretory signal sequence and a P10 promoter.

20 54. An expression vector comprising (a) a portion of a gene encoding a κ light chain constant region operatively linked to a human placental alkaline phosphatase secretory signal sequence and a polyhedrin promoter, and (b) a portion of a gene encoding an IgG₁ heavy chain constant region operatively linked to a honey bee melittin secretory signal sequence and a P10 promoter.

55. An expression vector comprising (a) a portion of a gene encoding a λ light chain constant region operatively linked to a human placental alkaline phosphatase secretory signal sequence and a polyhedrin promoter, and (b) a portion of a gene encoding an IgG₁ heavy chain constant region operatively linked to a honey bee melittin secretory signal sequence and a P10 promoter.

56. A vector comprising the nucleic acid sequence as set forth in SEQ ID NO:6.

57. A vector comprising the nucleic acid sequence as set forth in SEQ ID NO:7.

58. A vector comprising the nucleic acid sequence as set forth in SEQ ID NO:89.

59. A vector comprising the nucleic acid sequence as set forth in SEQ ID NO:90.

60. A vector comprising the nucleic acid sequence as set forth in SEQ ID NO:91.

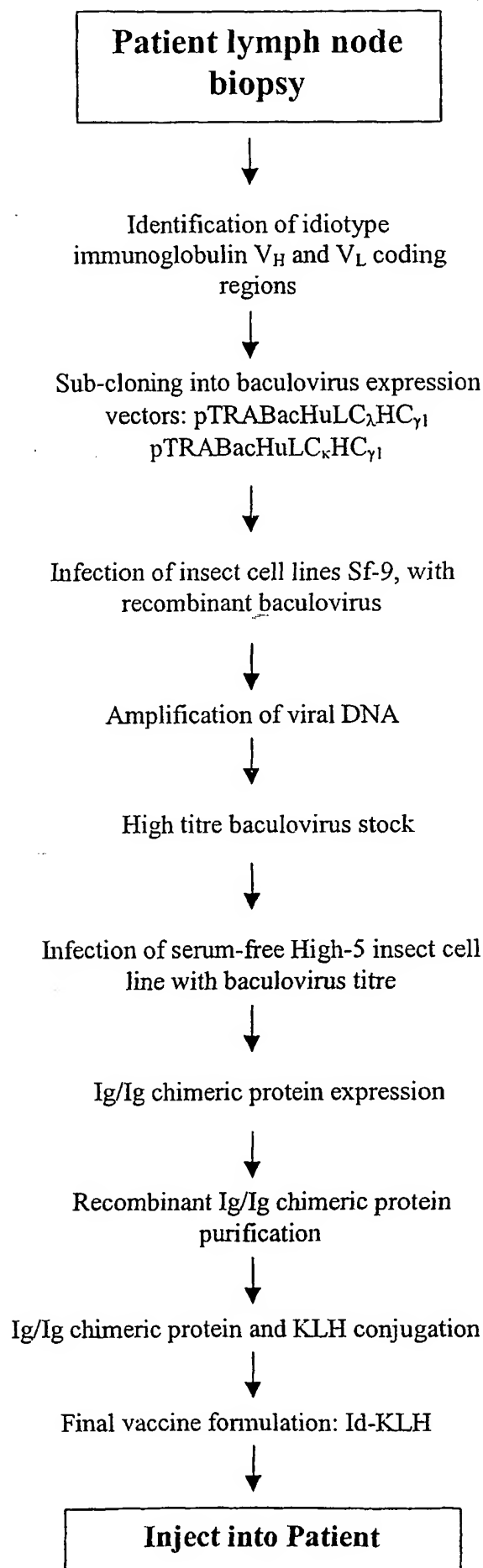
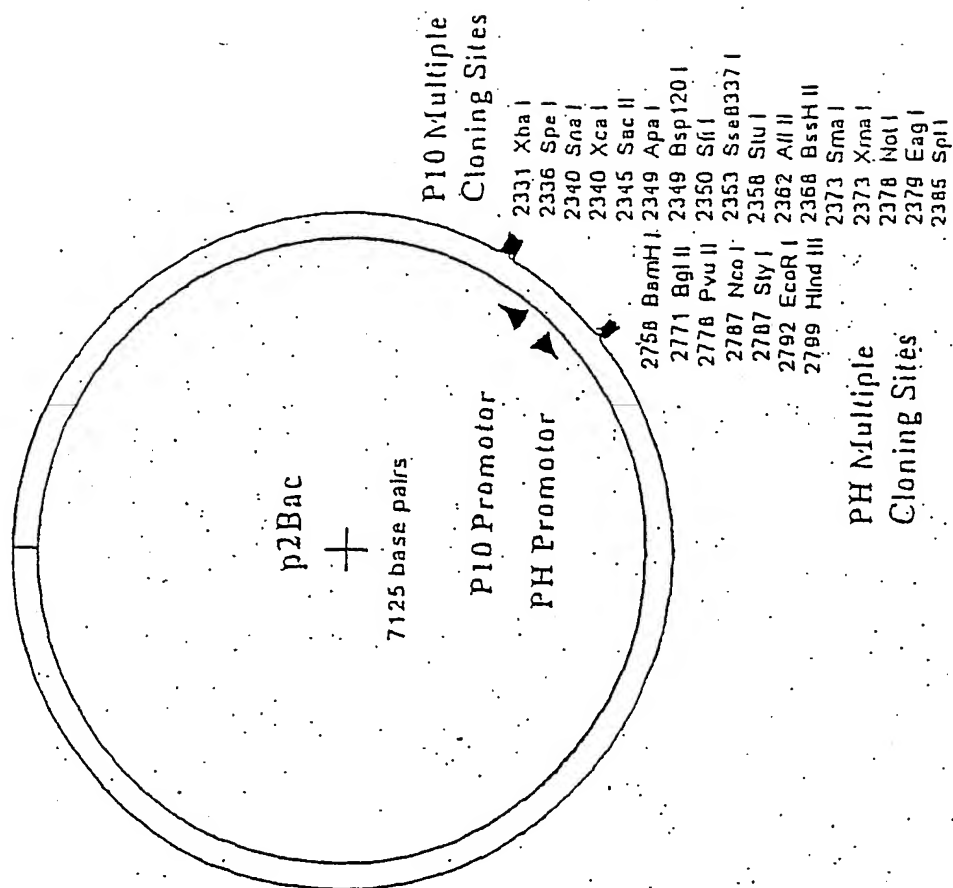


FIGURE 1



GGATTATTTCATACCGTCCACCATCGGGCGTGTAGCGGATCCGAGCTCGAGATCTGCGAGCTGGTACCATGGAAATTCGAA
GCTTGTCGTTGGATGGAAAGGAAAGAGTTCTACAGGGAAACTTGGACCCGCTTCAATGGAAAGACAGCTTCCCCCATTTGTTA
ACGACCAAGAAAGTATGATGTTTCTTGTGTTCAACATGCGTCCACCTAGACCCAAACCGTTGTTACAAATTCCTCGCC
CAACACGCTCTGCGTTGCGACCCCGACTATGTACCTCATGACGTGATAGGATCGTCGAGCCTTCATGGTGGGACAGCA
CAACGAGTACCGCATACGCTGGCTAAGAAAGGGCGGGCTGCCCAATAATGAACCTTCACCTCGGTACCGACTCTGCTGAAGAG
TCGAACAGTTCAJCGATCGTGTATCTGGGAGAACTTCTACAAAGCCCATCGTTACATCGGTACCGACTCTGCTGAAGAG
GAGGAATTCCTCTTGAAGTTTCCCTGGTGTTCARAAGTAAGGAGTTTGCACCAGAGCGACCTCTGTTCACTGGTCCGGC
GTATTAAACACAGATACATTTGTTATTAGTACATTTATTAAGCGCTAGATTTCTGTGCGTTGTTGATTTACAGACAAATTTGTT
GTACGTATTTTAATAATTGATTAAATTTATAATCTTTAGGGTGGTATGTTAGAGCGAANAATCAAAATGATTTTCAGCGCTCT
TTATACTGAATTTAAATATTAAATCCTCAATAGATTGTAAATAGGTTTCGATTAGTTTCNAACAAAGGTTGTTTTC
CGAACCGATGGCTGGACTATCTAATGGATTTTCGCTCAACGCCACAAACTTGCCNAATCTTGTAGCAGCAATCTAGCTT
TGTCGATATTCTGTTTGTGTTTGTAAATAAGGTTTCGACGTCTCAAAATATTATGCGCTTTTGTATTTCTTTCA
TCACGTGCTGTAGTGTACAAATTGACTCGACGTAAACACGTTAAATAAGCTAGCTTGGACATATTANACATCGGGCGTGT
TAGCTTTATAGGCCGATTATCGTGTGTCGCCAACCTCTGCTGTAGAAAGTTGCTTCGAAGACGATTTTGCCATAGCC
ACACGACGCCATTAAATTGTCGCTAACACGTCGCGATCAAAATTTGTAGTTGAGCTTTTGGAAATTAATTTCTGATTTG
CGGGCGTTTGGGGGGTTTCAATCTAATCTAATCTAATGCGGCGGTGGTGGAGCTGATGATAAATCTACCATCGGTGGAGGGCA
GTGGTAACATTTTCAGACGGCAATCTACTAATGCGGCGGTGGTGGAGCTGATGATAAATCTACCATCGGTGGAGGGCA
GGGGGGCTGGGGCGGAGGGGAGGGGAGGTGGTGGCGGCTGATGACAGACGGCGTTTAGGCTCAAAATGCTCTTTTAGG
CAACACAGTCCGACCTCAACTATTGTACTGGTTTGGGCGCGCTTTTGGTTTGACCGGTCTGAGACGAGTGGCAATTT
TTTCGTTTCTAATAGCTTCCAACAAATTTGTGCTGCTGTCTAAAGGTGACGCGGTTCGAGTTCCGTCGGCAATTTGGTGG
GCGGGCGCAATTCAGACATCGATGGTGGTGGTGGTGGAGCGCTGGAATGTTAGGCTCGGAGAGAGGTGGTGGCGG
CGGTGGCGCGGTTAAATTTGTTCTGGTTAGTTTGTTCGCGCACGATTGTGGCGACCGGCGGCGGCTGGCTGCA
CAACGGAAAGTCTGCTTCGAGGCGAGCTTGGGGTGGTGGCAATTCAAATATTAAATGGAAATCAAAATCGTAAANA
TCTGCTATAAGCATTTGTAATTTTCGCTATCGTTTACCGTGGCGATTTTAAACACCGCTCAATGTAAAGCAATTTGTA
AAGAGATTGCTCAAGCTCCGACGCGCGATACAAAGCTTTTCAATTTTACTACAGCATTTGTAGTGGCGAGACACCTTCGC
TGTCGTCGACTCGAGTTCATATAGTGTCACTAAATCGTATGTATGATACATAAGGTTATGATTAATTTAGTGGCGGT
TCTAACGACAAATATGTCCATATGGTGCACCTCTGAGTACAATCTGCTCTGATGCGGCAATAGTTAAGCCAGCCCGACACCC
GCCAACACCCGCTGACGGCGCTGACGGGCTTGTCTGCTCCGGCATCCGCTTACAGACAGCTGTGACCGCTCTCCGGGA
GCTGATGTGTCAGAGGTTTTCACCGTCAATCACCGAAGCGCGAGAGGAGGCTCGTGATACGCTATTTTATAG
GTTAATGTATGATAATAATGGTTTCTAGACGTACAGTGGCACTTTTCGGGGAATGTGCGGGAAACCCCTATTTGTT
ATTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAAATAACCTGATNAATGCTTCANATATTTGNAANAAGGA
AGAGTATGATATCAACATTTCCGTGTGCGCTTATTCCTTTTGGGGCAATTTTGCTTCCCTTCCCTTTTGGTCAACCA
GAAACGCTGGTGAAGTAAAGATGCTGAAGATCAAGTTGGGTGCACGAGTGGTTACATCGAACTGGATCTCAACAGCGG
TAAGATCCTTTGAGAGTTTTCGCCCGGAAGACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTAT
TATCCGCTATTGACGCCGGCAAGAGCACTCGGTCCCGCATACACTATTCAGAAATGACTTGGTTGAGTACTCAACCA
GTCACAGAAAGCATCTTACGGATGGCATGACAGTAAGAGAAATTAAGAGTGTGCTGCCATMCCATGAGTGTATACACTGC
GGCCAACTTACTTCTGACAAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTCACACAACTAGGGGATCATGTAACTC
GCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAACGACGAGCGGTGACACCGAGTGCCTGTAGCAATGGCA
ACAACGTTGCGCAACTATTAACTGGCGAAGTACTTACTCTAGCTTCCCGGCAACAAATTAATAGACTGGATGGAGGGCA

FIGURE 3B

TAAAGTTGCAGGACCACTTCTGGGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATTAANTCTGGAGCCGGTGAGCGTG
GGTCTCGCGGTATCATTGCAGCACATGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTTCAG
GCAACTATGGATGAAACGAAATAGACAGATCGCTGAGATAGGTGCCCTCACTGATTAAAGCATTGGTANCTGTCAGACCCNAGT
TTACTCATATATACCTTTAGATTGATTTAAACTTTCATTTTAAATTTAAAAGGATCTAGGTGAAGATCCCTTTTGTATATC
TCATGACCAAAATCCCTTAAACGTGAGTTTTCGTTCCACTGAGCGTACAGCCCGTAGNAAGAATCAAAAGGATCTTCTTTGA
GATCCCTTTTTCGCGGTAACTCTGCTGCTTGCATAACAAACCCGCTACCAAGCGGTGGTTTGTTTGCCGGATCA
AGAGCTACCAACICTTTTCCGAGGTAACTGGCTTCAGCAGAGCGCAGATACCAATATCTGTCCCTTCTAGTGTAGCCGT
AGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCTACATACCTCGCTCTGCTAATCTGTACCAGTGGCTGCTGCC
AGTGGCGATAAGTCTGTCTTACCGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAAACGGG
GGGTTCTGTGCACACAGCCAGCTTGGAGCGAACGACCTACACCGAAGTGAATACCTACAGCGTGAGCATTTGAGNAAGCG
CCACGCTTCCCGAAGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCGAGGTCGGAAACAGGAGAGCGCAGGAGGCTT
CCAGGGGGAACGCCCTGGTATCTTTATAGTCTTGTCTGGGTTTCGCCACCTCTGACTTGAGCGTCCGATTTTGTGTGCTC
GTCAGGGGGGCGGAGCCTATGGAAACCGCCAGCAACGCGGCTTTTACGGTTCCCTGGCTTTTGTGCTGCTTTTGTGCTC
ACATGTTCTTTCCCTGCTTATCCCTGATCTGTGGATAACCGTATTACCGCTTTTGAGTGAGCTGATACCGCTCGCCGC
AGCGGAACGACCGAGCGCAGGTACGTAGCGGAGGAGCGGAAGAGCGGCCCAATACGCAAAACCGCTCTCCCCGCGCG
TTGGCCGATTTCATTAATGCAGGTTAACCTGGCTTATCGAATTAATACGACTCACATATAGGGAGNCCGGCAGATCGATCT
GTCGA

FIGURE 3C

Page 1 of 4

Figure 4 (Cont.)

CCCCAGAAATAGAACCTACTCAGACAAATGCGATGCAATTTCTCTCATTTTATTAGGAAAAGGACAGTGGGAGTGGCAC
CTTCCAGGGTCAAGGAAGCACGGGGAGGGGCAACAAACAGATGGCTGGCAACTAGAAAGGACACAGTCCAGGCTGATCAG
CGAGCTCTAGCTAGACTATTATTTACCCGGAGACAGGAGAGGCTCTTCTCGGTGTAGTGGTTGTGACAGAGCCCTCATGC
ATCAGGAGCATGAGAAGACGTTCCCTGCTGCTCCACCTGCTCTTGTCCACGGTGTAGCTTGTCTGTAGAGGAAGAAAGGAGCC
GTCCGAGTCCAGCACGGGAGGGTGGTCTTGTAGTTGTCTCCGGCTGCCCATTTGCTCTCCACCTCCACGGCGATGTCGC
TGGGATAGAACCTTTGACCAAGGAGTCAAGGCTGAGCTGGTTCTTGGTCAGCTCATCCCGGATGGGGGACGGGTGTAC
ACCTGTGGTTCTCGGGGCTGCCCTTTGGCTTTGGAGATGGTTTCTCGATGGGGCTGGGAGGGCTTTGTTGGAGACCTT
GCACTTGTACTCTTGCATTGAGCCAGTCTTGGTGCAGGACGGTGTAGGACGCTGACACACAGGTACGTGCTGTGTACT
GCTCTCCCGGGCTTTGTCTTGGCATTAAGCACTTCCACGGCTCCACGTACCACTTGAACCTTGACCTCAGGGTCTTCG
TGGCTCAGCTCCACCAACCGCATGTGACCTCAGGGTCCGGGAGATCATGAGGTGTCTTGGGTTTGGGGGGAAGAG
GAAGACTGACGGTCCCCCAGGAGTTCAAGTGGCGGTGGGATGTGTAGTTTGTGTCACAAGATTTGGGCTCAACTTTCT
TGTCCACCTTGGTGTGTGGCTTGTGATTACGTTGCAGATGTAGGTCTGGGTGCCCAAGCTGTGAGGGCACGGTC
ACCACGCTGTGAGGAGTAGAGTCTGAGGACTGTAGGACAGCCGGGAAGTGTGACCGCCGCTGGTGTGAGGGCCCTGA
GTTCCACGACACCGTCAACGGTTCCGGGAAGTAGTCTTACACAGGACGCCAGGGCCGCTGTGCCCCCAGAGGTGCTCT
TGGAGGAGGTGCCAGGGGAAGACCGATGGGCCCTTGGTGGAGGCTGAGGAGACGGTGTACCAAGGTTCCCTGGCCCCAG
GAGTCAAGTAGTAGTGGGCCAGCCACTGTTTCCCGCTTTCGCACAGTAATAAACGGCCGTGTCTCGGCTCTCAGGCT
GTTCAAGTGCAGATATAGCGTGTTCATGGAATGTCTCTGGAGATGGTCAATCGGCCCGTCAACGGAGTCTGCATAATATG
TGGTAGTTCTCTAGCACTAATAGCCGACCCACTCCAGCCCCATCCCTGGAGCTTGGCGGACCCAGCTCATGGCATAG
CTGCTAAAGCTGAATCCAGAGGCTGCACAGGAGTCTCAGGACCCCCCAGGCTGTACCAAGCTCCCCAGACTGCAC
CAGCTGCACCTCGTCCGCATAGATGTAAAGAAATGTACAGCACTAATAAACTAGTGCACAGTTGACTAAGAATTTTCATGC
GGCCGCGTACGATTGTAAATAAAATGTAATTTACAGTATAGTATTTTAATTAATATACAAATGATTTGATAATAATCTT
ATTAACTATAATATATTGTGGTGAATTAAGGTCCTCGCATCCCAAGCTTGGCGTTTGGCGTTTATTTCTTGTCTCGA
GTAAAGTAGCGTATTCTGAATCTTTGTAAATAAGCACACAGGACTCCAAACGCTTGGCGTTTATTTCTTGTCTCGA
GGATATCATGGAGATAATTAATAATGATAACCACTCGCAATAATAAGTATTTTACTGTTTTCGTAAACAGTTTGTAAAT
AAAAAACCTATAAATATCCGGATTATTATACACCTCCACCACTCGGGCGTGTAGCGGATCCATGGTGGACCCCTGCA
TGCTGTCTGTCTGTCTGTAGGCTGTAGGCTACAGCTCTCCCTGGGCAATCGACATCCAGATGACCCAGTCTCCATCC
TCCCTGTCTGTCTGTAGGAGACAGAGTCACTTGTCCGGGCAAGTGTAGAGTATTAGACCTATTATAAATTGGTA
TCAGCAGAAACAGGGAAAGCCCTTAACCTCTGTATCTATTATGCAACCAATTTGCAAGTGGGTCCTCCATCAAGGTTCA
GTGGCAGTGGATCTGGGACAGATTTTCACTCTCACCATCAGCAGTGTGCAACCTTGGAATTTTGGGACTTATTATTGTCAA
CAGAGTTCCAAACACCGTCACTTTCCGGCCCTGGGACCAAGTGGATATGAAGACTGTGGCTGCACCAAGTGTCTTCATCTT
CCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCTGTGAATAACTTCTATCCAGAGAGGCCA

Figure 4 (Cont.)

AAGTACAGTGGAAAGTGGATAACGCCCTCCAAATCGGGTAACTCCAGGAGAGTGTCAAGAGCAGGACAGCAAGGACAGC
 ACCTACAGCCTCAGCAGCACCCCTGACCGTGAGCAAGCAGACTACGAGAAACACAAAGTCTACGCCCTGCCAAGTCAACCA
 TCAGGGCTGAGCTCGCCCTGTCACAAAGAGCTTCAACAGGGAGAGTGTAAATAGAGCTTGTCTGTTGGATGGAAGGAA
 AAGAGTTCTACAGGGAACCTTGGACCCGCTTCAATGGAAGACAGCTTCCCATTTGTTAACGACCAAGAAAGTGTGATGTT
 TTCCCTTGTGTCAACATGCGTCCCACTAGACCCCAACCGTTGTACAAATTCCTGGCCCAACACGCTCTGCGTTGCGACCC
 CGACTATGTACCTCATGACGTGATTAGGATCGTCGAGCCTTCACTGAGTACACCAACTCGTTCGAAACAGTTCATCGATCGTGC
 CTAAGAAGGGCGGCTGCCCAATAATGAACCTTCACTGAGTACACCAACTCGTTCGAAACAGTTCATCGATCGTGC
 ATCTGGAGAACTTCTACAGCCCATCGTTTACATCGGTACCGACTCTGCTGAAGAGGAGAAATTCCTCTGAAGTTTC
 CCTGGTGTCAAAAGTAAAGGAGTTTGACACGACGCACTCTGTTCACTGGTCCGGCGTATTAAACACAGTACATTTGTT
 ATTAGTACATTTATTAAGCGGTAGATTCTGTGCGTTGTTGATTACAGACAATTTGTTGACGTAATTTTAATAATTCATTA
 AATTTATAATCTTTAGGGTGGTATGTTAGAGCGAAATCAAAATGATTTTCAGCGTCTTTTATATCTGAATTTAAATATTAA
 ATCCTCAATAGATTGTTAAATAGGTTTCGATTAGTTTCAAAACAAAGGTTGTTTCCGAACCGATGGCTGACTATCTA
 ATGGATTTCGCTCAACGCCACAAAACCTTGCAAAATTTGAGCGCAATCTAGCTTTGTGCGATATTCGTTTGTGTTTG
 TTTTGTAAATAAGGTTGACGTGTTCAAAATATTATGCGCTTTTGTATTCTTCACTGCTGTTAGTGTACAATTG
 ACTGACGTAAACACGTTAAATAAAGCTAGCTTGGACATAATTAAACATCGGGCGTGTAGCTTTATTAGGCCGATTATCG
 TCGTCTGCCAACCTCGTCTGTAGAGTTGCTTCCGAAGACGATTTTGCATAGCCACACGACGCTATTAAATTGTGTC
 GGCTAACACGTCGCGGATCAAAATTTGATGTTGAGCTTTTGGAAATTTATCTGATTGCGGGCGTTTGGCGGGTTTCA
 ATCTAACTGTGCCGATTTTAAATTCAGACAAACACGTTAGAAAGCAGTGTGAGCGGTGGTAAACATTTTCAGACGGCAA
 TCTACTAAATGGCGGCGGTGTGAGCTGATGATAAATCTACATCGGTGGAGCGCAGCGGGCTGGCGCGGAGCGGG
 AGCGGAGTGTGGCGGTGTGACAGACGGCGGTTTGGCTCAATGCTCTTTAGGCAACACAGTCCGACCTCAACTA
 TTGTAAGTGTTCGGCGCGCTTTTGGTTTGACCGGTCTGAGACGAGTGCATTTTTCGTTTCTAATAGCTTCCAAAC
 AATTGTTGTCGTCTAAAGGTGACGGGTTGAGGTTCCGTCGGCATTTGGTGGAGCGGCGCAATTCAGACATCGA
 TGGTGGTGGTGGAGCGCTGGAATGTTAGGACCGGAGAAAGGTGTTGGCGGCGGTGCCCGGTATAATTTGTT
 CTGGTTTAGTTTGTCCGCGACGATTGTTGGGCAACCGGCGAGCGCGCTGGCTGCAACACGGAAGGTCGCTGCTCGA
 GGCAGCGCTTGGGTGGCAATTCATAATTAATAATGGAATACAAATCGTAAATACTGCTATAAGCATTTGTAATTT
 GCTATCGTTTACCGTCCGATATTTAAACACCGCTCAATTTGTAATTTGTAAGAGATTTGTCAGCTCCGCA
 CGCCGATAACAAGCCTTTTCAATTTTACTACAGCATTTGATGTCGAGACACTTCGCTGTCGACTCGAGTTCTATAG
 TGTACCTAAATCGTATGTATGATACATAAGGTTATGATTAATTTAGCGCGTTCTAACGACAAATATGTCATATG
 GTGCACCTCTCAGTACAATCTGCTCTGATGCGCATAGTTAAGCCAGCCCGACACCCGCAACACCCGCTGACGCGCCCT
 GACGGCTTGTCTGCTCCCGCATCCGCTTACAGACAAAGTGTGACCGTCTCCGGAGCTGCAATGTGTAGAGGTTTCA
 CCGTCAACACCGGAGAGGAAAGGCGCTCGTGATACGCCCTATTTTATAGGTTAAATGTGATGATAATAATGTT

Figure 4 (Cont.)

TTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGGGAAACCCCTATTGTGTTATTTTCTAAATACATTCAAATA
TGATCCGCTCATGAGACAAATAACCTGATAAATGCTTCAATAATTAAGAAAGGAGAGTATGAGTATTCACACATTTC
CGTGCGCCCTTATCCCTTTTTCGGGCATTTTGCCCTTCTGTTTTCCTCAACCCAGAAAACGCTGGTGAAGTAAAGA
TGCTGAAGATCAGTTGGGTGCACGAGTGGTTACATCGAATGGAATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCC
CCGAGAACGTTTTCGAATGATGAGCACTTTTAAAGTTCTGCTATGTGCGCGGTATTTATCCGTATTTGACGCCGGCAA
GAGCAACTCGTCCCGCATACATACTCTCAGAAATGACTTGGTTGAGTACTCACAGTCAAGAAAAGCATCTTACCGGA
TGGCATGACAGTAAGAGAAATATGCAAGTGTGCCATAACCATGAGTGAATAACA CTGCGGCCAACTTACTTCTGACAAACGA
TCGGAGGACCGAAGGAGCTAACCGCTTTTTCACAAACATGGGGATCATGTAACTCGCCTTGATCGTTGGGAAACCGGAG
CTGAATGAAGCCATACCAACGACGAGCGGTGACACCAAGATGCCCTGTAGCAATGGCAACAACGTTGCGCAACTATTAAAC
TGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAAATAGACTGGATGGAGGGGATAAAGTTGCAGGACCACTTCTGC
GCTCGGCCCTTCCGGCTGGCTGGTTTATGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTTGCAGCA
CTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGAGTCAGGCAACTATGGATGAACGAAATAG
ACAGATCGCTGAGATAGGTGCCCTCACTGATTAAGCATTGGTAACGTGCAGACCAAGTTTACTCATATATACCTTAGATTG
ATTTAAAACTTCAATTTTAAATTAAGGATCTAGGTGAAGATCCTTTTGTAAATCTCATGACCAAAATCCCTTAAACGT
GAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTCTGCGCGTAAT
CTGCTGCTTGCAAAACAAAAAACCAACCGCTACAGCGGTGGTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTCCGA
AGGTAACTGGCTTCAGCAGAGCGCAGATACCAATACCTGTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAAC
TCTGTAGCACCGCTACATACCTCGCTCTGCTTAATCCTGTTACAGTGGCTGCTGCCAGTGGCGATAAGTCTGTCTTAC
CGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTGAAACGGGGGTTCTGTGCACACAGCCAGCT
TGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAAGATGAGAAAGCCACGCTTCCGAGGGGAAACGCTGATCT
GCGGACAGGTATCCGGTAAGCGGACAGGTTCGAAACAGGAGAGCGACGAGGAGCTTCCAGGGGGGAAACGCTGATCT
TTATAGTCTGTGCGGTTTCGCCACCTCTGACTTGAAGCTTCGATTTTGTGATGCTCTGTCAGGGGGGCGGAGCTATGGA
AAAACGCCAGCAACGGGGCTTTTACGGTTCTGCGCTTTTGTGCTCACATGTTCTTCTGCGTTATCC
CCTGATTTCTGTGGATAACCGTATTAACCGCTTTGAGTGAAGTATACCGCTCGCGGAGCGGAAACGAGCGGAGCGGA
GTCAAGTGAAGGAGGAGCGGCAATACGCAACCGCTCTCTCCCGCGGCTTGCGCGGATTCATTAATGCAGGT
TAACCTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCGGCAGATCGATCTGTCTGA

FIGURE 5A

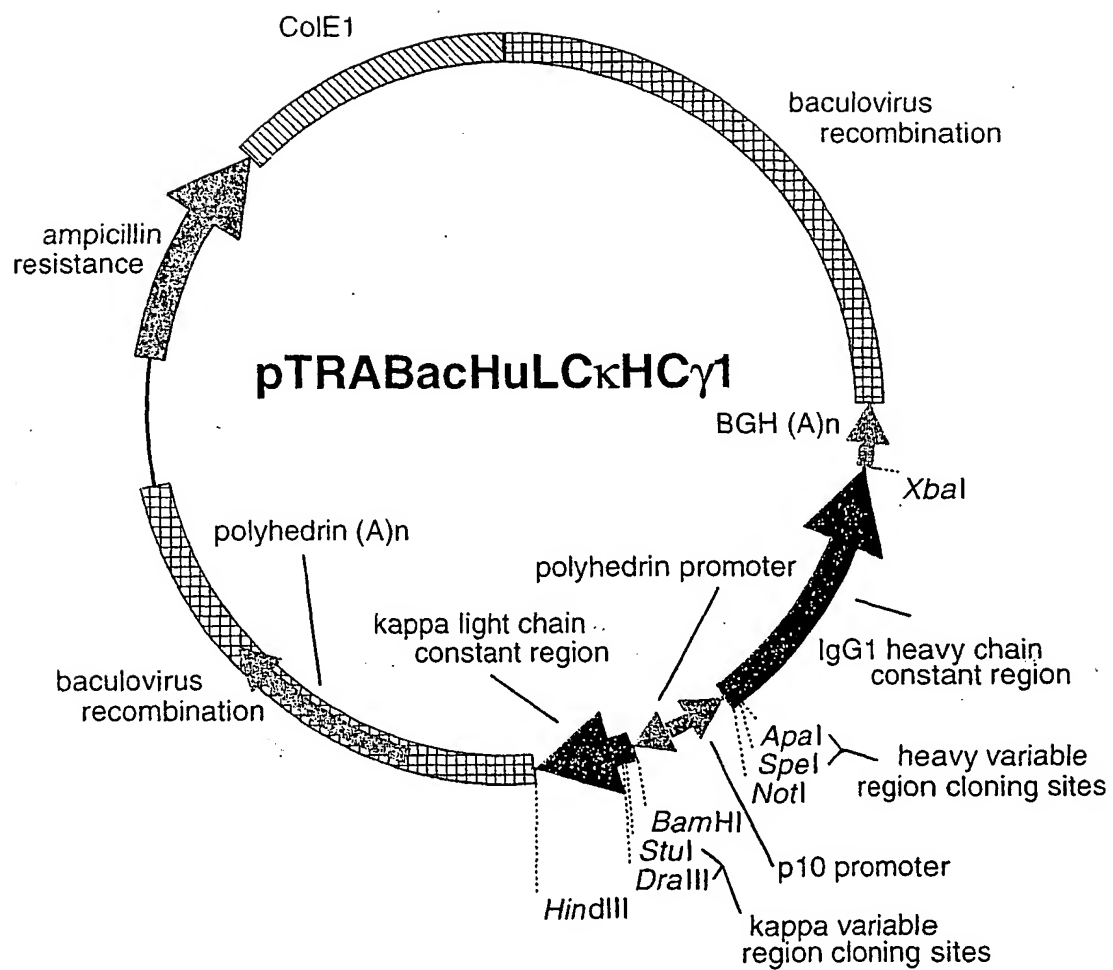
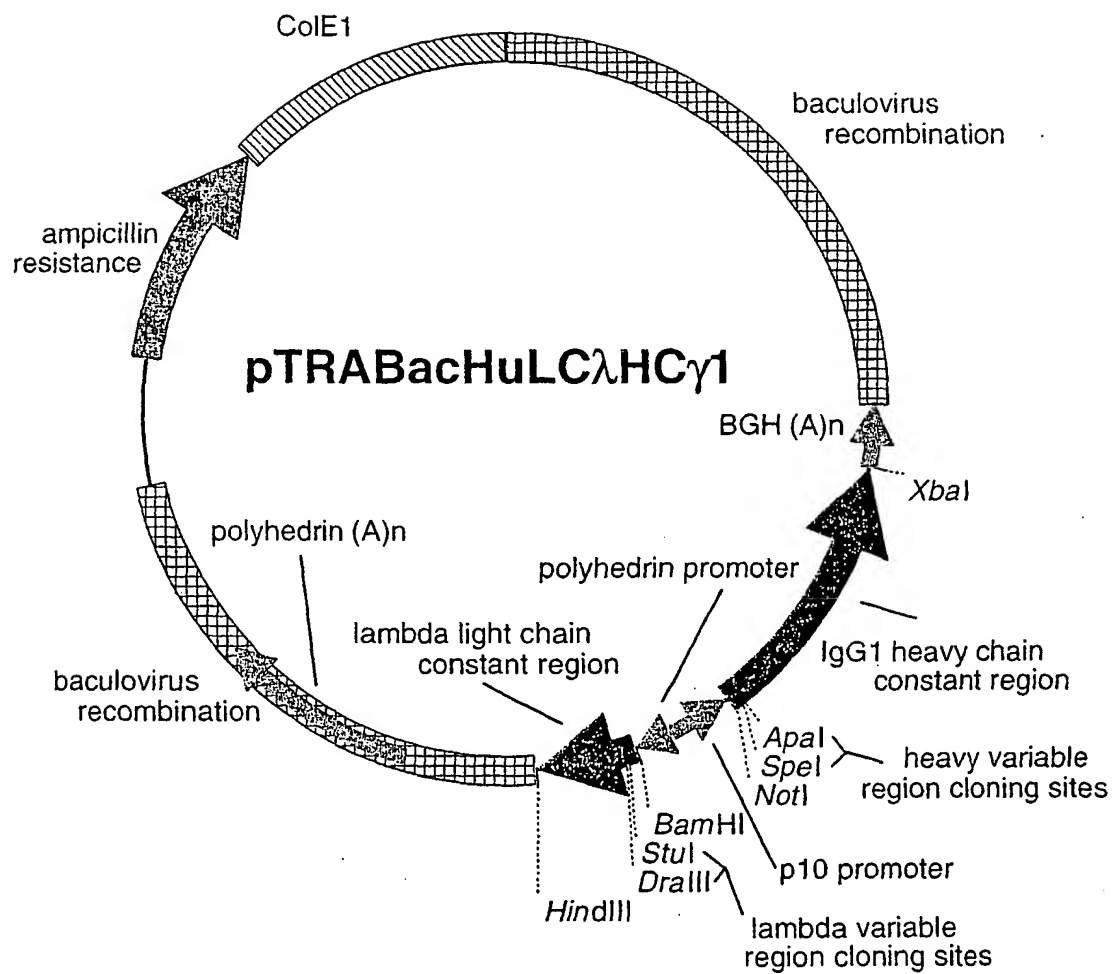


FIGURE 5B



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FIGURE 6A **pTRABacHuLCkHCλ1 DNA Sequence**

GCAGTTCGTTGACGCCCTTCCTCCGTGTGGCCGAACACAGTCGAGCGGGTGGTCGATGACCAGCGGCGTGCACGCGACGACAAAGTAT
CTGTACACCGAATGATCGTGGGCGGAAGCACGTCGGCCCTCCAAAGTGGCAATATTGGCAAAATTCGAAAAATATATACAGTTGGGTTGTTTT
GGCATAATCTATCGTGGCGTTGGGCATGTACGTCGAAACGTTGATTGGCATGCAAGCCGAAATTAAATCATTCGCGATTAGTGGGATTAA
AACGTTGTACATCCTCGCTTTTAAATCATGCGCTCGATTAAATCGGCAATCGAGTCAAGTGATCAAAAGTGTGGAATAAATGTTTTCTTTG
TATCCCGAGTCAAGCGCAGCGCGTATTTTAAACAACTAGCCATCTTGTAGTTAGTTTCATTTAATGCAACTTTATCCAAATAATATAT
TATGTATCGCACGTCAAGAAATTAAATGCGCCCGTTGTGCGCATCTCAACACGACTATGATAGAGATCAAAATAAGCGCGAATTAAATA
GCTTGGACGCAACGTGACGATCTGTGCAACGCGTTCCGGCACGAGCTTTGATTGTAATAAGTTTTTACGAAGCGATGACATGACCCCC
GTAGTGACAAACGATCAGCCCAAAAGAACTGCCGACTACAAAAATTACCGAGTATGTCGGTGACGTTAAACCTATTAAGCCATCCAAATCG
ACCGTTAGTCGAATCAGGACCGCTGGTGCAGAACCGCGAAGTATGGCGAATGCATCGTATAAACGTTGTGGAGTCCGCTCATTAGAGCG
TCATGTTTAGACAAAGAAAGCTACATATTAAATTGATCCCGATGATTTTATTGATAAATTGACCCCTAACTCCATACACGGTATTTCTACAA
TGGCGGGGTTTTTGGTCAAAATTTCCGGACTGCGATTGTACATGCTGTAAACGGCTCCGCCCACTATTAAATGAAATTAAAAATTTCCAATT
TTAAAAAACGCAGCAAGAGAAACATTTGTATGAAAGAAATCGGTAGAAGGAAAGAAAAATGTCTGCGACATGCTGAACAACAAGATTAAAT
ATGCCCTCCGTGTATAAAAAAAATATTGAACGATTTGAAAGAAAAACAATGTACCGCGCGGGTATGTACAGGAAGAGGTTTATACTAAA
CTGTTACATTGCAAAACGTGGTTTCGTGTGCCAAGTGTGAAAACCGATGTTTAAATCAAGGCTCTGACGCAATTTCTACAACCCAGACTCCA
AGTGTGTGGGTGAAGTCATGCACTCTTTTAAATCAAAATCCCAAGATGTGTATAAACCAACCAAACTGCCAAAAAATGAAAACTGTCGACAAG
CTCTGTCCGTTTGTGGCAACTGCAAGGGTCTCAATCCTATTTGTAATTATTGAATAATAAAACAATTATAAATGCTAAATTTGTTTTT
TATTAACGATACAAACCAACGCAACAAACATTTGTAGTATTATCTATAATTGAAACGCGTAGTTATAATCGCTGAGGTAATAATTT
AAAAATCATTTTCAAAATGATTACAGTTAAATTTGCGACAATATAAATTTTATTTTACATAAACTAGACGCGCTTGTCTCTTCTTCGT
ATTCTTCTCTTTTTCATTTTCTCTCATAAAAATTAACATAGTTATTATCGTATCCATATATGTATCTATCGTATAGAGTAAATTTT
TTGTTGTCATAAAATATATATGCTTTTAAATGGGTGTATAGTACCGCTGCGCATAGTTTTCGTGTAATTTACAAACAGTCTATTTTC
TGGTAGTTCTTCGGAGTGTGTTGCTTTAAATTATTAATTAATAATCAATGAATTTGGGATCGTCGGTTTTGTACAAATATGTTGCCGG
CATAGTACGAGCTTCTTCTAGTTCAATTACACCAATTTTGTAGCAGCACCGGATTAACATAAATTTTCCAAAAATGTTGTACGAACCGTTA
AACAAAAACAGTTACCTCCCTTTTCTATACTATTGTCTGCGAGCAGTTGTTTGTGTTGTTAAATAAAGCAATGTTGTAATGAGACGCAC
AAACTAATATCACAAACTGGAATGTCTATCAATATATAGTTGCTGATATCTCCCCAGCATGCCCTGCTATTGTCTTCCCAATCCTCCCC
CTTGCTGTCCTGCCCCACCCCAAGATAGAAATGACACCTACTCAGACAAATGCGATGCAATTTCCCTCATTTTATTAGGAAAGGA

[illegible]

Figure 6A (Cont.)

CAAATGATTTTCAGCGTCTTTATATCTGAATTTAAATATTAAATCCTCAATAGATTGTGTAATAATAGGTTTCGATTAGTTTCAAAACAAGG
GTTGTTTTTCCGAACCGATGGCTGGACTATCTAATGGATTTTCGCTCAACGCCACAATACTTGCCAAATCTTGTAGCAGCAATCTAGCT
TTGTCGATATTTCGTTTGTGTTTTTGTAAATAAAGGTCGACGCTCAAAATATTATGCGCTTTTGTAATTTCTTTTCATCACTGTC
GTTAGTGTAACAATTGACTCGACGTAAACACGTTAAATAAAGCTAGCTTGGACATATTAAACATCGGGCGTGTAGCTTTATTAGGCCGA
TTATCGTCGTCCCAACCTCGTCGTTAGAGTTGCTTCCGAAGACGATTTTGCCATAGCCACACGACGCCATTAAATTGTGTGCGG
TAACACGTCGCGGATCAAAATTTGTAGTTGAGCTTTTGGAAATTAATTTCTGATTGCGGGCGTTTGTGGCGGGTTTCAATCTAACTGTGCG
CCGATTTTAATTAGACAAACACGTTAGAAAGCGATGGTCAGGCGGTGTAACATTTAGACGGCAAAATCTACTAATGGCGCGGTGGT
GGAGCTGATGATAAATCTACCATCGGTGGAGCGCAGCGGGGCTGGCGGAGCGGAGGCGGTGGTGGCGGTGATGCAGACGG
CGGTTTAGGCTCAATGTCTCTTTAGGCAACACAGTCGGCACCTCAACTATTGTACTGGTTTCGGGCGCCGTTTGTGGTTGACCGGTC
TGAGACGAGTGCGAATTTTTTCGTTTCTAATAAGCTTCCAAACAATTGTTGTCTGTCTAAAGTGACGCGGTTGAGGTTCCGTCGGC
ATTGGTGGAGCGGGCGCAATTCAGACATCGATGGTGGTGGAGCGCTGGAATGTTAGGCACGGGAGAAAGTGGTGGCGG
CGGTGCCGCGGTATAATTTGTTCTGTTTAGTTTGTTCGCGCACGATTTGGGCAACCGCGCAGCGCGCTGGCTGCACAACGGAAG
GTCGCTGCTTCGAGGCGCGCTTGGGGTGGTGGCAATTCAATATTAAATTGGAATACAAATCGTAAATACTGCTATAAGCATTTGTA
ATTTCGCTATCGTTTACCGTGCCGATATTAAACACCGCTCAATGTAAGCAATTGTATTGTAAGAGATTGTTCTCAAGCTCCGCAACGCC
GATAACAAGCCTTTTCATTTTACTACAGCATTTGAGTGGCGAGACACTTCGCTGTCTGCTGAGTTCTATAGTGTCACTTAATC
GTATGTGATGATACATAAAGTTATGTAATTAATTGTAGCCGCTTCTAACGACAATAATGTCCATATGGTGCACCTCTCAGTACAAATCTGC
TCTGATGCCGCATAGTTAAGCCAGCCCGACACCCCGCTGACGCGCCCTGACGGCTTGTCTGCTCCCGGCATCCGCTTA
CAGACAAGCTGTGACCGTCTCCGGAGCTGCTGTCAGAGGTTTTCACCGTCAACCGCAACCGCGGAGAGAAAGGCTTCGTGA
TACGCCATTTTTTATAGGTTAATGTCAATAATAATGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAAACCCCT
ATTGTTTTTCTAAATACATTCAATAATGTAATCCGCTCATGAGACAATAACCTGATAAATGCTTCAATAATATTGAAAAAGGAA
GAGTATGAGTATCAACATTTCCGTGTCGCCCTTATTCCTTTTGTGGGCAATTTTGCCCTTCTGCTTTCACCCAGAAACGCTGG
TGAAAGTAAAGATGCTGAAGATCAGTTGGGTGCAGAGTGGTTACATCGAATGATCTCAACAGCGGTAAGATCCTTGAGAGTTTT
CGCCCCGAGAACGTTTTTCCAAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGGGTATTATCCCGTATTGACGCCGGCAAGACA
ACTCGGTCGCCGATACATACTTCTCAGAAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAAGCATCTTACGGATGGCATGACAGTAA
GAGAAATTATGAGTGTGCCATAACCATGAGTGATAACACTCGCGCCAACTTACTTCTGACAAACGATCGGAGGACCGAAAGGAGCTAACC
GCTTTTTTGACAAACATGGGGGATCATGTAACTCGCCCTTGATCGTTGGAAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGA
CACCACGATGCTGTAGCAATGGCAACAACGTTGCGCAACTATTAACTGCGCAACTACTTACTCTAGCTTCCCGGCAACAATTAATAG
ACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAAATCTGGAGCCGCT

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Figure 6A (Cont.)

GAGCGTGGGTCTCGCGGTATCATTTGCAGCATGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGC
AACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCCTCACTGATTAAGCAATTGGTAACTGTCTAGACCAAGTTTACTCATATA
TACTTTAGATGATTTAAACTTTTAAATTTAAAGGATCTAGGTGAAGATCCCTTTTGTGATAATCTCATGACCAAAATCCCTTAAC
GTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAAGGATCTTCTTGAGATCCCTTTTCTGCGCGTAATCTGCTGC
TTGCAAAACAAAAAACCCAGCTACCCAGCGGTGTTTGTGTCGGATCAAGAGCTACCAACTCTTTTCCGAAGGTAACTGGCTTCAG
CAGAGCGCAGATACCAAATACTGCTCTTAGTGTAGCCGTAGTTAGGCCACCCTTCAAGAACTCTGTAGCACCGCTTACATACCTCG
CTCTGCTAATCCTGTACCAAGTGGCTGCTGCCAGTGGCGATAAGTCGTGCTTACCGGGTTGGA CTCAAGACGATAGTTACCGGATAAG
GCCAGCGGTCGGGCTGAACGGGGGTTCTGTGCAACAGCCAGCTTGGAGCGAACCTACACCGAACTGAGATACCTACAGCGTGA
GCATTGAGAAAGCGCCACGCTTCCGAAGGGAGAAAGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGAGAGCGCACGAGGG
AGCTTCCAGGGGAAACGCCCTGGTATCTTTATAGTCCCTGTGCGGTTTCCGCACCTCTGACTTGAGCGTCGATTTTGTGATGCTCGTCA
GGGGGGCGGAGCCCTATGGAAAAACGCCAGCAACGGGCCCTTTTACGGTTCCCTGGCCCTTTTGTCTGCTCAGATGTTCTTTCC
TGCGTTATCCCCGTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCGAGCCGAAACGACCGAGCGCAGCG
AGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAAACCGCCTCTCCCGCGCGTTGGCCGATTTCATTAAATGCAGGTTAACCTGG
CTTATCGAAATTAAATACGACTCACTATAGGGAGACCGGCAGATCGATCTGTCTGA

GCAGTTCGTTGACGCCCTTCCTCGTGTGGCCGAACACGTCGAGCGGGTGTGATGACCAGCGGGTGTCCGCGACGCGACCAAG
TATCTGTACACCGAATGATCGTGGGCGAAGGCACGTCGGCCTCCAAAGTGGCAATATTGGCAAAATTCGAAAAATATATACAGTTGGG
TTTTGTTGGCATATCTATCGTGGCGTTGGCATGTACGTCCGAACGTTGATTTGCATGCAAGCCGAAATTAATCATTTGCGATTAG
TGCGATTAAAACGTTGTACATCCTCGCTTTTAAATCATGCGTCGATTAAATCGGCAATCGAGTCAAGTGATCAAAAGTGTGGAATA
ATAGTTTTCTTTGTATTCCCGAGTCAAGCGCAGCGGTATTTTAAACAACTAGCCATCTTGTAAAGTTAGTTTCATTTAAATGCAACTT
TATCCAAATAATATATTATGTATCGACGCTCAAGAAATTAACAATGCGCCCGTTGTGCGCATCTCAACACGACTATGATAGAGATCAAA
TAAAGCGGAATTAAATAGCTTGGACGCAACGTGCACGATCTGTGCACGCTTCGGCACGAGCTTTGATTGTAATAAGTTTTTTTA
CGAAGCGATGACATGACCCCGTAGTGACAAACGATCACGCCCAAAGAACTGCCGACTACAAAATTACCGAGTATGTCGGTGACGT
TAAAACTATTAAAGCCATCCCAATCGACCGTTAGTCCGAATCAGGACCGCTGGTGCAGAAAGCCGGAAGTATGGCGAATGCATCGTAT
AAACGCTGTGGAGTCCGCTCATTAGAGCGTCAATGTTTAGACAAAGAAAGCTACATATTTAAATTGATCCCGATGATTTTATTGATAAAATT
GACCCCTAACTCCATACACGGTATTTCTACAATGGCGGGTTTTGGTCAAAATTTCCGGACTCGGATTTGTACATGCTGTTAAACGGCTC
CGCCCACTATTAAATGAAATTTAAAAATTTCCAAATTTAAAAAACGCAGCAAGAGAAAAATTTGTATGAAAGAAATGCGTAGAAGGAAAG
AAAAAATGTCGTCGACATGCTGAAACAAAGATTAAATATGCCTCCGTGTATAAAAAAAATATTGAAACGATTTGAAAGAAAAACAATGT
ACCGCGCGCGGTATGTACAGGAAGAGGTTTATACTAACTGTTTACATTGCAAAACGTGGTTTTCGTGTGCCAAGTGTGAAAAACCGCAT
GTTTAAATCAAGGCTTGACGCATTTCTACAACCAAGCTCCAAAGTGTGGGTGAAGTCATGCATCTTTTAAATCAAAATCCCAAGAT
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TTTGTAAATTATTGAAATAATAAAACAAATTATAAATGCTAAAATTTGTTTTTTTATTAAACGATACAAACCAACGCAACAAACATTTGT
AGTATTATCTATAATTGAAAAACCGGTAGTTATAATCGCTGAGGTAATAATTAAAAATCAATTTTCAAATGATTCACAGTTAAATTTGCG
ACAAATAAAATTTTATTTTACATAAACTAGACGCCCTTGTGCTCTTCTTCTCGTATTCCTTCTTTTTTTCATTTTCTCCTCATAA
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AAATGGGTGTATAGTACCGCTGCGCATAGTTTTTCTGTAATTTACAACAGTGCATATTTTCTGGTAGTTCTTCCGAGTGTGTGCTTT
TAAATTATAAATTATATAAATCAATGAATTTGGGATCGTCGGTTTTTGTACAATAATGTTGCCGCGCATAGTACGACGCTTCTTCTAGT
TCAATTACACCAATTTTGTAGCAGCACCGGATTAAACATAACTTTCCAAAATGTTGTACGAAACCGTTAAACAAAAACAGTTTCACTCC
CTTTTCTATACTATTGTCTGCGAGCAGTTGTTTGTGTTAAAAATAACAGCCATTGTAATGAGACGACAAAACTAATATCAAAAC
TGGAAATGTCTATCAATATATAGTTGCTGATATCTCCCCAGCATGCCCTGCTATTGCTTTCCCAATCCTCCCTTGTGCTGCTGCC

CCACCCACCCCCAGAAATAGAAATGACACCTACTAGACAAATGCGATGCAATTTCTCTCATTTTATTAGGAAAGGACAGTGGGAGTGC
GCACCTTCAGGGTCAAGGAAAGGCACGGGGAGGGGCAAAACAAACAGATGGCTGGCAACTAGAAAGGCACAGTCGAGGCTGATCAGCG
AGCTCTAGTCTAGACTATTATTTACCCGGAGACAGGGAGAGGCTCTTCTGCGTGTAGTGGTGTGTCAGAGCCTCATGCAATCACGGA
GCATGAGAAAGACGTTCCCTGCTGCCACCTGCTCTTGTCCACGGTGAGCTTGTGTAGAGAAAGAAAGAGCCGTCGGAGTCCAGCA
CGGAGGGCTGGTCTTGTAGTTGTTCTCCGGCTGCCATTGCTCTCCCACTCCACGGCGATGTCGCTGGGATAGAAGCCTTTTGACC
AGGCAGGTCAGGCTGACCTGGTCTTTGGTTCAGCTCATCCCGGATGGGGCAGGGTGTACACCTGTGGTCTCTCGGGCTGCCCCCTTT
GGCTTTGGAGATGGTTTTCTCGATGGGGCTGGGAGGGCTTTGTTGGAGACCTTGCACCTTGTACTCCTTGCCATTTCAGCCAGTCCCT
GGTGCAGGACGGTGAGGACGCTGACCAACACGGTACGTTGCTGTTGTACTGCTCCTCCCGCGCTTTGTCTTTGGCAATTATGCACCTCC
ACGGCTCCACGTACCGATTGAACCTTGACCTCAGGGTCTTCTGGCTCAGCTCCACCACCGCATGTGACCTCAGGGGTCCGGGA
GATCATGAGGGTGTCTTTGGGTTTTTGGGGGAAGAGGAAGACTGACGGTCCCCCAGGAGTTCAGGTGCTGGGCACGGTGGGCATG
TGTGAGTTTGTTCACAAAGATTGGGCTCAACTTTCTTGTCCACCTTGTGGTGTGCTGGGCTTGTGATTCACGTTGCAGATGTAGGTC
TGGGTGCCAAGCTGCTGGAGGCAACGGTCACACGCTGCTGAGGGAGTAGAGTCTTGAGGACTGTAGGACAGCCGGGAAGGTGTG
CAGCCCGCTGGTCAGGGCGCTGAGTTCACGACACCGTCACCGGTTCCGGGAAAGTAGTCTTGACCAGGACAGCCAGGGCCCGCTG
TGCCCCAGAGGTGCTCTTGGAGGAGGTGCCAGGGGAAGACCGATGGGCCCACTAGTGCAACGTTGACTAAAGAAATTTTCATGCGG
CCGGTACGATTGTAAATAAAATGTAAATTTACAGTATAGTATTTTAAATTAATAACAATCATAGTCCCTTGTGTAAAGTATGATCGCTA
ATAATATAATTGTGTGGTTGAATTAAGGTCCCGCATCCTCAAAATGCAATAATCATAGTCCCTTGTGTAAAGTATGATCGCTA
TTTCTGAAATCTTTGTAAAAATAGCACACAGGACTCCAAACGGCTTTGGCGTTTTTATTTCTTGTCTGAGGATATCATGGAGATAAATTA
AAATGATAAACCATCTCGCAAAATAAATAAGTATTTACTGTTTTCGTAAACAGTTTGTAAATAAAAAAACCTATAAATATTCCGGATT
ATTCTATACCGTCCACCATCGGGCGTCTAGCGGATCCATGGTGGGACCTTGCACTGCTGCTGCTGCTGCTGCTGCTAGGCTCACC
CAGTGTCACTCTGTTCCCGCCCTCCTCTGAGGAGCTTCAAGGCCAACAGGCCACACTGGTGTGCTCTCAATAAGTGACTTCTACCCGG
GAGCCGTGACAGTGGCCTGGAAAGCAGATAGCAGCCCGTCAAGCGGGAGTGGAGACCAACACCCCTCCAAACAAAGCAACAAC
AAGTACCGCGCCAGCAGCTACCTGAGCCTGACCGCTGAGCAGTGGAAAGTCCCAAAAAGCTACAGCTGCCAGGTACGCAATGAAAG
GAGCACCGTGGAGAGACAGTGGCCCTTACAGAAATGTTTCAATAGTAAAAAGCTTGTGCTGGATGAAAAAGAAAAAGAGTTCTACAGGG
AAAACTGGACCCGCTTCATGGAAGACAGCTTCCCCATTGTTAAACGACCAAGAGTGAATGGATGTTTTCTTGTGTCAACATGCGT
CCCACTAGACCCAAACCGTTGTACAAATTCCTGGCCCCAACACGCTCTGCGTTGGCACCCCGACTATGTACCTCATGACGTGATTAG
GATCGTCGAGCCTTCATGGGTGGGCAGCAACAAACGAGTACCGCATCAGCCTGGCTAAGAAAGGCGGGCTGCCCAATAATGAAC
TTCACTCTGAGTACACCAACTCGTTTCGAACAGTTTCATCGATCGTGTCACTCTGGGAGAACTTCTACAAAGCCCATCGTTTACATCGGT
ACCGACTCTGCTGAAGAGGAGGAAAATTCTCCTTGAAGTTTCCCTGGTGTTCAAAAAGTAAAGGAGTTTGCACCCAGACGCACCTCTGTT
CACTGGTCCGGCGTATTAAAAACACGATACATTTGTTATTAGTACATTTATTAAGCGCTAGATTCTGTGCGTTGTGATTTTACAGACA
ATTGTTGTACGTAATTTAAATAATTCAATTAATTTTAAATCTTTAGGGTGGTATGTTAGAGCGAAAAATCAAAATGATTTTTCAGCGTCT
TTATATCTGAAATTTAAATATTAATCTCTCAATAGATTTTGTAAAAATAGGTTTTCGATTGTTTCAAAACAAAGGGTTGTTTTTCCGAAC

Figure 6B (Cont.)

GATGGCTGACTATCTAATGGATTTTCGCTCAACGCCACAAAACCTTGCCAAATCTTGTAGCAGCAATCTAGCTTTGTGATATTCG
TTTGTGTTTTTGTAAATAAAGGTTTCGACGTCGTTCAAAATATATATGCGCTTTTGTATTTCTTTCATCACTGTCGTTAGTGATC
AATTGACTCGACGTAAACACGTTAAATAAGCTAGCTTGGACATATTTAAACATCGGGCGTGTAGCTTTATATAGGCCGATTTATCGT
CGTCGTCACAAACCTCGTTAGAGTTGCTTCCGAAGACGATTTTGCCATAGCCACACGACGCTATTAATTGTGTCGGCTAAC
ACGTCGCGATCAAAATTTGTAGTTGAGCTTTTGGAAATTAATTTCTGATTCGGGCGGTTTTCGAGGCTTCAATCTAACTGTGCC
CGATTTTAATTCAGACAAACACGTTAGAAAACGATGGTGACGGCGTGGTAACATTTTCAGACGGCAAACTCTACTAATGCGGCGGTG
GTGAGCTGATGATAAATCTACCATCGGTGGAGGCGCAGCGGGGCTGGCGGGAGGCGGAGGAGGTGGTGGCGGTGATGCA
GACGGCGGTTTAGGCTCAATGTCTCTTTAGGCAACACAGTCGGCACCTCAACTATTGTACTGTTTCGGGCGGCTTTTGTGTTT
GACCGGCTGAGACGAGTGCGATTTTTCGTTCTAATAGCTTCCAACAATTTGTGTCGTCTAAAGTGCAAGCGGTTGAG
GTTCCGTGCGCATTTGGTGAGCGGGCGGCAATTCAGACATCGATGGTGGTGGTGGAGCGCTGGAATGTTAGGCACGGGA
GAAGTGTTGGCGGCTGCCCGGCTATTAATTTGTTCTGTTTAGTTTGTTCGCGCACGATTTGTGGCACCGGCGCAGCGCGCCG
TGGCTGCACAAACGGAAGTCTGCTTCGAGGCGAGCTTGGGGTGGTGGCAATTAATTAATTTGGAATACAAATCGTAAA
AATCTGCTATAAGCATTTGTAATTTGCTTACGTTACCGTGCCGATATTTAAACAACCGCTCAATGTAAGCAATTTGTTGTAAAGA
GATTGCTCAAGCTCCGACGCCGATAACAAGCCTTTTCAATTTTACTACAGCAATTTAGTGGCGAGACACTTCGCTGTCGTCGAC
TCGAGTTCTATAGTGTACCTAAATCGTATGTGTATGATACATAAAGTTTATGTAATTTAGTCGCGCTCTAAACGACAAATATGT
CCATATGGTGCACTCTCAGTACAATCTGCTGTATGCCGATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCC
TGACGGGCTTGTCTGCTCCCGGCTACGCTTACAGACAAGCTGTGACCGCTCTCCGGAGCTGATGTGTCAGAGGTTTTCACCGTC
ATCACCGAAACGCGGAGAGGAAAGGCTCTGTGATACGCTATTTTATAGGTTAATGTGATGATAATAATGGTTTCTTAGACGT
CAGGTGGCACTTTTCGGGAAATGTGCGGGAACCCCTATTTGTTTATTTTCTAAATACATTTCAAAATATGTAATCCGCTCATGAGA
CAATAACCCCTGATAAATGCTTCAATAATATTTGAAAAGGAAGATAGTATGATATTCACCAATTTCCGCTGTCGCCCTTATTCCTTTT
TGCGGCATTTTGCTTCTGCTTCTGCTCAACCCAGAAACGCTGGTGAAGTAAAGATGCTGAAGATCAGTTGGTGCAACGAGTGG
GTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTCCCAATGATGAGCACTTTTAAA
GTTCTGCTATGTGGCGGATTAATCCCGTATTTGACCGCGGCAAGACAACCTGGTCCGCGCATACACTATTTCTCAGAATGACTT
GGTTGAGTACTCACCAAGTACAGAAAAGCATCTTACGGATGGCATGACAGTAAAGAAATATGCAAGTGTGCCATAAACCATGAGTG
ATAACACTGCGGCCAATCTTCTGACAAACGATCGGAGGACCGAAGAGCTAACCGCTTTTTCACAACATGCGGGATCATGTA
ACTCGCCTTGATCGTTGGAAACCGGAGCTGAATGAAGCCATACCAACGACGAGCGTGACACCAACGATGCCCTGTAGCAATGGCAAC
AAGTTGGGCAACTATACTAGCGGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGCGGGATAAAGTTG
CAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTTGCTGATAAACTGGAGCCGGTGAGCGTGGGTCTCGCGGTATC
ATTGCAGCACTGGGCGCAGATGTTAAGCCCTCCCGTATCGTAGTTATCTACAGACGCGGGAGTCAGGCAACTATGGATGAACGAAA
TAGACAGATCGCTGAGATAGGTGCCCTCACTGATTAAGCATTTGTAACCTGTGAGACCAAGTTTACTCATATATACCTTTAGATTGATT
TAAAACTTCATTTTAAATTTAAAGGATCTAGGTGAAGATCCTTTTGTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCG

Figure 6B (Cont.)

TTCCACTGAGCGTCAGACCCCGTAGAAAAAGATCAAGGATCTTCTTGAGATCCTTTTTTTCTGCGGTAATCTGCTGCTTGCAAAAC
AAAAAAACCAACCGCTACCAAGCGGTGGTTTGTGTTGCCGGATCAAGAGCTACCAACTCTTTTTTCCGAAGGTAACTGGCTTCAGCAGAG
CGCAGATACCAATACTGTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCCTACATACCTCGCT
CTGCTAATCCTGTTACCAAGTGGCTGCTGCCAGTGGCGATAAGTCGTCTTACCGGGTTGGA CTCAAGACGATAGTTACCGGATAA
GGCGCAGCGGTCGGGCTGAACGGGGGTTTCGTGCACACAGCCAGCTTGAGCGGAACGACCTACACCGAACTGAGATACCTACAGC
GTGAGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGSCGACAGGTATCCGGTAAGCGGAGGGTCGGAACAGGAGAGCGC
ACGAGGGAGCTTCCAGGGGGAACGCCCTGGTATCTTTATAGTCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTGTG
ATGCTCGTCAGGGGGCGGAGCCCTATGGAAAAACGCCAGCAACGGGCCCTTTTACGGTTCCTGGCCCTTTTGCTGGCCCTTTTGCTC
ACATGTTCTTTCCTGCGTTATCCCTGATTCCTGTGGATAACCGTATTACCGCCCTTTGAGTGAGCTGATACCGCTCGCCGAGCCGA
ACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAACCGCCCTCTCCCCGCGGTTGGCCGATTCA
TTAATGCAGGTTAACCTGGCTTATCGAAATTAAACGACTCACTATAGGGAGACCGGCAGATCGATCTGTCTGA

FIGURE 6C
pTRABacHulCkHC γ 1 + Stuffers DNA Sequence

GCAGTTCGTTGACGCCCTTCCTCCGTGTGGCCGAACACGTCGAGCGGGTGGTCGATGACCGGCGGTGCCGACGCGACACAAG
TATCTGTACACCGAATGATCGTCGGCGAAGGACACGTCGGCCCTCCAAAGTGGCAATATTGGCAAAATTCGAAAAATATATACAGTTGGG
TTGTTTGGGCATATCTATCGTGGCGTTGGGCATGTACGTCGGAACGTTGATTGTCATGCAAGCCGAAATTAATCATTCGCGATTAG
TGCGATTAAAAACGTTGTACATCCTCGCTTTTAATCATGCGTCGATTAAATCGCGCAATCGAGTCAAGTGATCAAAAGTGTGGAATA
ATGTTTCTTTGTATTCCCGAGTCAAGCGCAGCGGTATTTTAAACAACTAGCCATCTTTGTAAGTTAGTTTCATTTAATGCAACTT
TATCCAATAATATATTATGTATCGCACGTCAAGAAATTAACAAATGCGCCCGTTGTGCGCATCTCAACACGACTATGATAGAGATCAAA
TAAAGCGCGAATTAATAGCTTTCGACGCAACGTCACGATCTGTGCACGCGTTCCGGCACGAGCTTTGATTGTAATAAGTTTITA
CGAAGCGATGACATGACCCCCGTAGTGACAAACGATCACGCCCAAGAACTCCGACTACAAAAATTACCGAGTATGTCGGTGACGT
TAAACTATTAAAGCCATCCAATCGACCGTTAGTCGAATCAGGACCGCTGGTGGAGAAAGCCGCGAAGTATGGCGAATGCATCGTAT
AACGTGTGGAGTCGCTCATTTAGAGCGTCAATGTTTAGACAAGAAAGCTACATATTAAATTGATCCGATGATTTTATTGATAAAATT
GACCTAACTCCATACACGGTATTCTACAAATGGCGGGGTTTGTGTCAAAAATTTCCGACTGCGATTGTACATGCTGTTAACGGCTC
CGCCCACTATTAAATGAAAAATTCCAATTTTAAAAAACGACGCAAGAGAAAAATTTGTATGAAAGAAATGCGTAGAAGGAAAG
AAAAATGTCGTCGACATGCTGAACAAACAGATTAAATATGCTCCGTGTATAAAAAAATATTGAACGATTTGAAAGAAAAACAATGT
ACCGCGCGGGGTATGTACAGGAAGAGGTTTATACATAAATCTGTTACATTGCAAAACGTTGGTTTCGTGTGCCAAGTGTGAAAAACCGAT
GTTTAAATCAAGGCTGTGACGCAATTTCTACAAACCAACGACTCCAAAGTGTGGGTGAAGTCATGCATCTTTTAAATCAAAATCCCAAGAT
GTGTATAAACCAACCAACTGCCAAAATAAGAAAACTGTGACAAAGCTCTGTCGTTTGTGTCGCAACTGCAAGGTCCTCAATCCTAT
TTGTAATTATTGAATAAATAACAAATTATAAATGCTAAATTTGTTTTTTATTAAACGATACAAACCAACGCAACCAAGAACATTTGT
AGTATTATCTATAATTGAACACCGGTAGTTATAATCGCTGAGGTAATAATTAAATCAATTTTCAAAATGATTCACAGTTAATTTGCG
ACAAATAAATTTTATTTTACATAAACTAGACGCCCTTGTCGTCTTCTTCGTATTTCTCTCTTTTCAATTTTCTCCTCATAA
AAATTAACATAGTTATTATCGTATCCATATATGTATCTATCGTATAGAGTAAATTTTTTGTGTGTCATAAATAATATATGTCTTTTT
AATGGGTGTATAGTACCGCTGCGCATAGTTTTTCTGTAATTTACAAACAGTCTATTTTTCTGGTAGTTCTTTCGGAGTGTGTGCTT
TAATATTAAAAATTTATATAATCAATGAATTTGGGATCGTCGGTTTTTGTACAATAATGTTGCCGGCATAGTACGAGCTTCTTCTAGT
TCAATTACACCATTTTTTAGCAGCACCGGATTAAACATAACTTTCCAAAATGTTGTACGAACCGTTAAACAAAAACAGTTCAACCTCC
CTTTTCTATACTATTGTCTGCGAGCAGTTGTTTGTGTTTAAATAACAGCCATTGTAATGAGACGCACAAACTAATATACAAAC
TGGAAAATGCTCTATCAATATATAGTTGCTGATATCTCCCCAGCATGCTGCTATTGTTCTTCCCAATCTCTCCCTTGTGCTGCTGCTGCC

Figure 6C (Cont.)

CCACCCACCCCCAGAAATGACACCTACTCAGACAAATGCGATGCAATTTCCCTCATTTTATTAGGAAAGGACAGTGGAGTG
GCACCTTCCAGGGTCAAGGAAGGCACGGGGAGGGGCAAAACAACAGATGGCTGGCAACTAGAAGGCACAGTCGAGGCTGATCAGCG
AGCTCTAGTCTAGACTATTATTACCCGGAGACAGGGAGAGGCTCTTCTGCGTGTAGTGGTTGTGCAGAGCCTCATGCAATCACGGA
GCATGAGAAGACGTTCCCTGCTGCCACCTGCTCTTGTCCACGGTGAGCTTGTGTAGAGGAAGAAAGGAGCCGTGCGAGTCCAGCA
CGGAGGCGTGGTCTTGTAGTTGTTCTCCGGCTGCCCATTTGCTCTCCACTCCACGGCGATGTCGCTGGGATAGAAAGCCTTTGACC
AGGCAGGTACGGCTGACCTGGTCTTGGTCAGCTCAATCCCGGATGGGGCAGGGTGTACACCTGTGGTTCTCGGGGCTGCCCTTT
GGCTTTGGAGATGGTTTCTCGATGGGGCTGGAGGGCTTTGTTGGAGACCTTGCACTTGTAACCTTGCCATTGAGCCAGTCCT
GGTGAGGACGGTGAGGACGCTGACCAACACGGTACGTGCTGTTGTACTGCTCCTCCCGGGCTTTGTCTTGGCATTAATGACCTCC
ACGCCGTCCACGTACCACTTGAACTTGACCTCAGGGTCTTCTGGCTCACGTCCACCAACACGCACTGTGACCTCAGGGGTCCGGGA
GATCATGAGGGTGTCTTGGGTTTGGGGGGAAGAGGAAGACTGACGGTCCCCCAGGAGTTTCAGGTGCTGGGCACGGTGGGCATG
TGTGAGTTTGTCAAGATTTGGGCTCAACTTTCTGTCCACCTTGGTGTGCTGGGCTTGTGATTCACGTTGCAGATGTAGGTC
TGGGTGCCCAAGCTGTGGAGGGCACGGTCAACGCTGCTGAGGGAGTAGAGTCTGAGGACTGTAGGACAGCCGGGAAGGTGTG
CACGCCGTGGTTCAGGGCGCTGAGTTCCACGACACCGTCAACGGTTTCGGGGAAGTAGTCTTTCAGCAGGACGCCAGGGCCGCTG
TGCCCCCAGAGGTGCTCTTGGAGAGGGTGCAGGGGGAAGACCGGATGGGCCCTTATCAAACTAGTGCAACGTTGACTAAGAAATTT
CATGCGGCCCGGTACGATTGTAAATAAAATGTAATTTACAGTATAGTATTTTAAATTAATAATACAAATGATTTGATAATAATTTCTTA
TTTAACTATAATATATTGTGTGGTTGAATTAAGTCCCGGCTCCCTCAAATGCATAATATCATAGTCCCTTGTGTAAAGTG
ATGCGTATTTCTGAATCTTTGTAAATAGCACACAGGACTCCAAACGGTTTGGCGTTTATTTTCTGCTCGAGGATATCATGGAG
ATAATTAAATGATAACCATCTCGCAATAAATAAGTATTTTACTGTTTTCGTAAACAGTTTGTAAATAAAACCTATAAAATATT
CCGGATTATTATACCCGTCCACCATCGGGCGTGTAGCGGATCCATGTTGGGACCCCTGCATGCTGCTGCTGCTGCTGCTGCTAGG
CCTTTGATAAACACCAAGTGTCTTCACTTCCCGGCTATCTGATGAGCAGTTGAAATCTGGAACCTGCCCTCTGTTGTGCTGCTGCTGAA
TAACTTCTATCCCGAGAGGCCAAAGTACAGTGGAAAGGTGATAACGCCCTTCCAAATCGGGTAACTCCCGAGGAGGTGTACAGAGC
AGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCTGACGCTGAGCAAGCAAGCAGACTACGAGAAACACAAAGTCTACGCCCTGC
GAAGTCAACCCATCAGGGCCTGAGCTCGCCCGTCAAAAGAGCTTCAACAGGGGAGAGTGTAAATAGAAGCTTGTGTTGGATGGAA
AGGAAAAGAGTTCTACAGGGAAACTTGGACCCGCTTTCATGGAAGACAGCTTCCCATTTGTAAACGACCAAGAGTGTGATGTTT
TCCTTGTGTCAACATGCGTCCCATAGACCCCAACCGTTGTACAAATTCCTGGCCCCAACACGCTCTGCTGCGACCCCGACTAT
GTACCTCATGACGTGATTAGGATCGTTCGAGCCTTCAATGGGTGGGAGCAACAACAGTACCGCATCAGCCTGGCTAAGAAGGGCGG
CGGCTGCCCCAATAATGAACCTTCACTCTGAGTACACCAACTCGTTTCGAACAGTTTCATCGATCGTGTCTCTGCGGAGAACTTCTTACA

AGCCCATCGTTTACATCGGTACCGACTCTGCTGAAAGAGGAGGAAATTCCTCTGAAGTTTCCTCTGGTGTTCAAAAGTAAAGGAGGAGTTT
GCACCAGACGCACCTCTGTTCACTGGTCCGGCGTATTAAAAACACGATACATTTGTTATTAGTACATTTATTAAAGCGCTAGATTCTGT
GCGTTGTTGATTTACAGACAAATTGTTGTACGTAATTTTAAATAATTCAATAAATTTATAACTCTTTAGGGTGGTATGTTAGAGCGGAAAA
TCAAATGATTTTTCAGCGTCTTTATATCTGAATTTAAATATTAAATCCTCAATAGATTTGTAAAAATAGGTTTCGATTAGTTTCAAAC
AAGGGTTGTTTTTCCGAAACCGATGGCTGGACTATCTAATGGATTTTTCGCTCAACGCCACAAAACTTGCCAAATCTTTGTAGCAGCAA
TCTAGCTTTGTGCGATATTCGTTTGTGTTTTGTTTGTAAATAAAGGTTTCGACGTCGTTCAAAATATTATGCGCTTTTGTATTTCCTTT
CATCACTGTCGTTAGTGTACAAATTGACTCGACGTAAACACGTTAAATAAAGCTAGCTTGGACATATTTAAACATCGGGCGTGTAGC
TTTATTAGGCCGATTATCGTCGTCCTCCAAACCTCGTCGTTAGAAATTGCTTCCGAAACGATTTTGCATAGCCACACGACGCC
TATTAAATTGTGTCGGCTAACACGTCGCGGATCAAAATTGTAGTTGAGCTTTTGGAAATTATTTCTGATTTGCGGGCGTTTTTGGGG
GGTTTCAATCTAACTGTGCCGATTTTAAATTCAGACAACACAGTTAGAAAGCGATGGTGCAGCGGTGGTAACATTTCAGACGGCAA
ATCTACTAAATGGCGCGGTGTTGAGCTGATGATAAATCTACCATCGGTGAGGCGCAGGCGGGGCTGGCGGCGGAGCGGAGGCG
GAGGTGTTGGCGGTGATGCAGACGGCGGTTTAGGCTCAAAATGCTCTTTAGGCAACACACAGTCGGCACCTCAACATATTGTACTGGTT
TCGGGCGCGTTTTTGGTTTGACCGGCTGAGACGAGTGCGATTTTTTTCGTTTCTAAATAGCTTCCAAACAATTGTTGTCGTCTGTC
TAAAGGTGCAGCGGTTGAGGTTCCGTCGGCATTTGGTGGAGCGGGCGCAATTCAGACATCGATGGTGGTGGTGGTGGAGGCGG
CTGGAATGTTAGGCACGGGAGAAAGTTGGTGGCGGTTGCCCGGTATAAATTGTTCTGGTTTAGTTTGTTCGCGCACGATTGTG
GGCACCGGCGCAGCGCCGCTGGCTGCACAACGGAAAGTCTGCTCTCGAGGCAGCGCTTGGGGTGGTGGCAATTCAATATTATA
ATTGGAATACAAATCGTAAAAATCTGCTATAAGCATTTGTAATTTTCGCTATCGTTTACCCTGCCGATATTAAACAACCGCTCAATGT
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GACACTTCGCTGTCGCTCGACTCGAGTTCTATAGTGTCACTTAATCGTATGTGTATGATACATAGGTTATGTAATAATTGTAGCC
GCGTCTTAACGACAAATATGTCATATGGTGCACCTCTCAGTACAATCTGCTCTGATGCCGATAGTTAAGCCAGCCCCGACACCCCGC
CAACACCCGCTGACCGCCCTGACGGGCTTGTCTGCTCCCGCATCCGCTTACAGACAAAGCTGTACCGCTCTCCGGAGCTGCATG
TGTCAGAGGTTTTTCAACCGTCAATCACGAAACGCGGAGAGGAAAGGCCCTCGTGATACGCCATTTTTTATAGGTTAATGTCAATGAT
AATAATGGTTTTCTTAGACGTGAGTGGCACTTTTTCGGGGAAATGTGCGGGAAACCCCTATTTTGTTTATTTTTCTAAATACATTCAA
ATATGTAACCGCTCATGAGACAAATAACCTGATAAATGTTCAATAAATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCCGT
GTCGCCCTTATTCCCTTTTTTTCGGGCATTTTGCCCTTCCCTGTTTTTGTCTCACCCAGAAACGCTGGTGAAAGTAAAAAGATGCTGAAGA
TCAGTTGGGTGCACGAGTGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTTCGCCCGGAAGAACGTTTTTC
CAATGATGAGCACTTTTAAAGTTCTGCTATGTTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTGCGCGCATA

Figure 6C (Cont.)

CACTATTCTCAGAAATGACTTGTTGAGTACTCACCAAGTCACAGAAAAGCATCTTACGGATGCGATGACAGTAAGAGAAATTATGTCAG
TGCTGCCATAAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAAACGATCGGAGGACCGAAGAGCTAACCGCTTTTTTTCG
ACAAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAAACGACGAGCGTGACACCAACG
ATGCCGTAGCAATGGCAACAACGTTGGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTG
GATGGAGCGGATAAAGTTGCAGGACCACCTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTG
AGCGTGGGTCTCGCGGTATCATTTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAG
GCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCCTCACTGATTAAGCATTTGGTAACTGTACAGCCAAAGTTTACTC
ATATATACTTTAGATTGATTTTAAAACTTCAATTTTAAATTTAAAGGATCTAGGTGAAGATCCTTTTGTGATAAATCTCATGACCAAAA
TCCCTTAACGTGAGTTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAAGGATCTTCTTGAGATCCTTTTTTCTGCGC
GTAACTCTGCTGTTGCAAAACAAAACCAACCGCTACACGCGGTGGTTTGTGTGCGGATCAAGAGCTACCAACTCTTTTCCGAA
GGTAACCTGGCTTCAGCAGAGCGCAGATACCAATACTGTCTCTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAG
CACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCACTGGCTGTGCGAGTGGCGATAAGTCGTCTTACCGGGTTGGACTCA
AGACGATAGTTACCGGATAAGGCGCAGCGGTGCGGCTGAACGGGGGTTCTGTGCACACAGCCAGCTTGGAGCGAACGACCTACAC
CGAACTGAGATACCTACAGCGTGAGCATTGAGAAAGCGCCACGCTTCCGGAAGGAGAAAAGCGGACAGGTATCCGGTAAGCGGCA
GGTTCGGAAACAGGAGAGCGGACGAGGGAGCTTCCAGGGGGAACCGCTGGTATCTTTATAGTCTGTCGGGTTTCGCCACCTCTGA
CTTGAGCGTTCGATTTTGTGATGCTCGTCAGGGGGCGGAGCCCTATGGAAAAACGCCAGCAACGCGGCCCTTTTACGGTTCCTGGC
CTTTTGTGCTGGCCTTTTGTCTCAGATGTTCTTCTGCGTTATCCCTGATTTCTGTGATAACCGTATTAACCGCCTTTTGTGAGTGA
ATACCGCTCGCCGACGCCGAACGACCGAGCGCAGGTGAGTGAAGGAGGAGCGCCCAATACGCAAAACCGCCTCTC
CCCGCGCGTTGGCCGATTTCATTAAATGCAGGTTAACCTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCGGCAGATCGAT
CTGTCTGA

pTRABacHuLC λ HC γ 1 + Stuffers DNA Sequence

GCAGTTCGTTGACGCCCTTCCTCCGTGTGGCCGAACACGTCGAGCGGGTGGTCGATGACCGAGCGGGTGGCCGACGCGACGCGCAC
AAAGTATCTGTACACCGAATGATCGTGGGCGAAGGCAACGTCGGCCTCCAAAGTGGCAATATTGGCAATATCGAAAAATATATACAC
GTTGGGTGTTTGGCATACTATCGTGGCGTTGGGCATGTACGTCCGAACGTTGATTTGTCATGCAAGCCGAAATTAATCAT
TGCGATTAGTGGGATTAACCGTTGTACATCCCTCGCTTTAAATCATGCCGCGGTAAATCGCGCAATCGAGTCAAGTGATCA
AAAGTGTGGAATAATGTTTTCTTTTGTATTCCCGAGTCAAGCGCAGCGCGTATTTTAAACAACTAGCCATCTTTGTAAAGTTAGTTT
CATTTAATGCAACTTTATCCAAATAATATATTATGTCGACGTCAGAAATTAACAAATGCGCCCGTTGTGCGCATCTCAACACG
ACTATGATAGAGATCAATAAAGCGCGAATTAATATAGCTTGGCAGCGCAACGTGACCGATCTGTGCAACGCTTCGGCACGAGC
TTTTGATTGTAATAAGTTTTTACGAAGCGATGACATGACCCCGCTAGTGACAAACGATCACGCCCAAAGAACTGCCGACTACAA
AAATTACCGAGTATGTCGGTGACGTTAAAACTATTAAGCCATCCAATCGACCGTTAGTCGAATCAGGACCGCTGGTGGGAGAA
CCCGCGAAGTATGGCGAATGCATCGTATAACGTGTGGAGTCCGCTCATTTAGAGCGTCATGTTTAGACAAAGAAAGCTACATATTT
AAATTGATCCCGATGATTTTATTGATAAAATTGACCCTAATCCCATACACGGTATTTCTACAATGGCCGGGTTTTTGGTCAAAAATTT
CCGGA CTGCGATTGTACATGCTGTTAACGGCTCCGCCCACTATTAAATGAAATTAATAAATTCCAATTTTAAAAAACGCGACGAA
AGAAACATTTGTATGAAAGAAATCGTAGAAGGAAAGAAAAATGTGTCGACATGCTGAACAACAAGATTAAATATGCTCTCCGTG
TATAAAAAAATATTGAACGATTTGAAAGAAAAACAATGTACCGCGCGGTATGTACAGGAAGAGGTTTATACATAAACTGTT
ACATATTGCAACCGTGGTTTCGTGTGCCAAGTGTGAAAAACCGATGTTTAAATCAAGGCTCTGACGCAATTTCTACAAACCGACTCC
AAAGTGTGTGGTGAAGTCATGCATCTTTTAAATCAATCCCAAGATGTGTATAAACCAACCAACTGCCAAAAAATGAAAACTGT
CGACAAGCTCTGTCCGTTTGTGGCAACTGCAAGGCTCTCAATCCTATTGTAAATTATTGAATAATAAAACAAATTATATAATGC
TAAAAATTTGTTTTTATTAAACGATACAAACCAACGCAACAGAACATTTGTAGTATTATCTATAATTGAAAAACGCGTAGTTAT
AAATCGCTGAGGTAAATTTAAATCATTTTCAAATGATTCACAGTTAAATTGCGACAAATATAAATTTTATTTTACATAAACTA
GACGCCCTGTCTCTTCTTCGTATTCTCTTTTTCATTTTCTCTCATAAAAAATAACATAGTTATTATTCGTATCC
ATATATATGATCTATCGTATAGAGTAAAAATTTTTTGTGTCTATAAAATATATATGTCTTTTTAAATGGGGTGATAGTACCGCTGC
GCGATAGTTTTTCTGTAAATTACAAACAGTGCATTTTTCTGGTAGTTCTTCGGAGTGTGTGCTTTAAATTATTAATTTATATAA
TCAATGAAATTTGGGATCGTCGGTTTTGTACAAATATGTTGCCGGCATAGTACGACGCTTCTTCTAGTTTCAATTACACCATTTTT
TAGCAGCACCGGATTAAACATAACTTTCCAAAAATGTTGTACGAACCGTTAAAAACAAAAACAGTTTCACTCCCTTTTCTATACTAT
TGTCTGCGAGCAGTTGTTGTTGTTAAATAAACAGCCATTGTAAATGAGACGCAACAACTAATATACAAACTGGAAATGCT

Figure 6D (Cont.)

ATCAATATATAGTTGCTGATATCTCCACGATGCCGTGCTATTGTCTTCCCAATCCTCCCCCTTGCTGTCTGCCCAACCCCA
CCCCCCAGAAATAGATGACACCTACTCAGACAAATCGATGCAATTCCTCAATTTATTAGGAAGGACAGTGGAGTGGCACC
TTTCCAGGTCAGGAAGGACCGGGGAGGGCAAAACAACAGATGGCTGGCAACTAAAGGCACAGTCGAGGCTGATCAGCGAG
CTCTAGTCTAGACTATTATTACCCGGAGACAGGGAGAGGCTCTTCTGCGTGTAGTGGTTGTGCAGAGCCTCATGCTATCACGG
AGCATGAGAAAGACGTTCCCTGCTGCCACCTGCTCTTGTCCACGGTGAGCTTGTCTGTAGAGGAAGAGGAGCCGTGCGAGTCC
AGCACGGGAGGCGTGGTCTTGTAGTTGTTCTCCGGCTGCCCATTTGCTCTCCACTCCACGGCGATGTGCTGGGATAGAAAGCC
TTTTTGACCCAGGCAGGTGAGCTGACCTGGTTCTTGGTCAGCTCATCCCGGATGGGGCAGGTGTACACCTGTGGTTCTCGGG
TGCTGCCCTTTGGCTTTGGAGATGGTTTCTCGATGGGGCTGGGAGGCTTTGTTGGAGACCTTGCACTTGTACTCTCCTTGCCCA
TTTTTAGCCAGTCTGTTGTCAGGACGGTGAGGACGCTGACCAACACGGTACGTGCTGTGTGTACTGCTCCTCCCGCGGCTTTGTCTT
GGGCATTATGCACTTCCACCGCGTCCACGTACCAGTTGAACCTTGACCTCAGGGTCTTTCGTGGCTCACGTCACCAACCAACGCATG
TGACCTCAGGGTCCGGGAGATCATGAGGTTGCTTGGGTTTTGGGGGAAGAGGAAGACTGACGGTCCCCCCAGGAGTTCA
TGCGGTGCTGGGCAACGGTGGCATGTGTGAGTTTGTGCAAGATTGGGCTCAACTTTCTTGTCCACCTTGGTGTGTGCTGGGCTT
GTGATTACAGTTGCAGATGTAGGTCTGGGTGCCCAAGCTGCTGGAGGGCACGGTCAACACGCTGCTGAGGGAGTAGAGTCCCTG
AGGACTGTAGGACAGCCGGGAAGGTGTGCACGCCGTGGTCAGGGCGCCTGAGTTCCACGACACCGTCAACCGGTTTCGGGGAAG
TAGTCTTGTACCAGGCAGCCAGGGCCGCTGTGCCCCCAGAGGTGCTCTTGGAGGAGGGTGCCAGGGGAAGACCGGATGGGCC
CTTATCAAACTAGTGCAACGTTGACTAAGAAATTCATGCGGCCCGGTACGATTGTAAATAAAATGTAAATTTACAGTATAGTAT
TTTAAATTAAATATACAAATGATTTGATTTCTTATTTAACTATAATATATTGTGTGTGGTTGAAATTAAGGTCCCCGGCAT
CCTCAATATGATAATATCATAGTCCCCCTTGTGTAAAGTGTGCGTATTTCTGAATCTTTGTAAAAATAGCACACAGGACTCCA
ACGCGCTTTGGCGTTTATTTCTTGTCTCGAGGATATCATGGAGATAAATTAAAAATGATAACCATCTCGCAAAATAAATAAGTATT
TTTACTGTTTTCGTAAACAGTTTGTAAATAAAAAACCTATAAAATATTCGGGATTATTATACACCGTCCCACCATCGGGCGTGTCTA
CGCGGATCCATGTTGGACCCCTGCACTGCTGCTGCTGCTGCTAGGCCCTTGATAACACCCAGTGTCACTCTGTCTCCCCG
CCTCCTCTGAGGAGCTTCAAGCCCAACAGGCCACACTGGTGTGTCTCTATAAGTGACTTCTACCCGGGAGCCGTGACAGTGGCC
TGGAAGGCAGATAGCAGCCCGCTCAAGCGGGAGTGGAGACCAACACACCTCCAACAAGCAACAACAAGTACGCGGCCAG
CAGCTACCTGAGCCTGACCGCTGAGCAGTGGAAAGTCCACAAAAGCTACAGCTGCCAGGTACCGCATGAAGGGAGCACCCGTGG
AGAAAGACAGTGGCCCCCTACAGAAATGTTTCATAGTAAAAAGCTTGTGCTTGGATGGAAGGAAAAAGAGTTCTACAGGGAAAACTTGG
AACCCGCTTCATGGAAAGACAGCTTCCCATTTGTTAAACGACCAAGAGTGTGATGTTTTCTTGTGTCAACATGCGTCCCCAC
TAGACCCAAACCGTTGTTACAAATTCTTGGCCCCAACACGCTCTGCGTTGGACCCCGACTATGTA CCTCATGACGTGATTAGGA

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Figure 6D (Cont.)

TCGTCGAGCCTTCATGGTGGGAGCAACACGAGTACCGCATCAGCCTGGCTAAGAGGGCGGGCTGCCCAAATAATGAAC
CTTCACCTGAGTACACCAACTCGTTTGAACAGTTCATCGATCGTGTCTATCTGGAGAACTTCTACAAGCCCATCGTTTACAT
CGGTACCGACTCTGCTGAAGAGGAGGAAATTCCTTGAAGTTTCCCTGGTGTTCAAAAGTAAAGAGTTTGACACGAGCGCAC
CTCTGTTCACTGGTCCGGGTATTAACACGATACATTTGTTATAGTACATTTATTAAGCGCTAGATTCTGTGCGTTGTTGA
TTTACAGACAATTGTTGTACGTATTTTAAATAATTCATTAATTTATAATCTTTAGGGTGGTATGTTAGAGCGAAATCAAATG
ATTTTCAGCGCTCTTTATATCTGAATTTAAATATTAATCTCAATAGATTGTTGTAATAAGTTTCGATTAGTTTCAAAACAAGG
GTTGTTTTTCCGAACCGATGGCTGGACTATCTAATGGATTTTCGCTCAACGCCACAAAACCTTGCCAAATCTTTGTAGCAGCAAT
CTAGCTTTGTGATATTCGTTTGTGTTTGTGTTTGTAAATAAGGTTTCGACGTCGTTCAAAATATATATGCGCTTTTGTATTTCT
TTCATCACTGTCGTTAGTGTAACAATTGACTCGACGTAACACGTTAAATAAAGCTAGCTTGACATATTTAAACATCGGGCGTG
TTAGCTTTATTAGCGGATTAATCGTCGTCGTCCTCCAAACCTCGTCGTTAGAAAGTTGCTTCGGAAGACGATTTTGCCATAGCCAC
ACGACGCTATTAATTTGTGTCGGCTAACACGTCGCGATCAAAATTTGTAGTTGAGCTTTTGGAAATTTATTTCTGATTTGCGGGC
GTTTTTGGCGGGTTTCAATCTAACTGTGCCCCGATTTTAAATTCAGACAACACGTTAGAAAGCGATGGTGCAGGCGGTGGTAAC
ATTTTCAGACGGCAATCTACTAATGCGGGCGGTGGTGAGCTGATGATAAATCTACCATCGGTGAGGCGCAGCGGGGCTGG
CGCGGAGGCGGAGGAGGTGGTGGCGGTGATGCAGACGGCGGTTTAGGCTCAAATGTCTCTTTAGGCAACACAGTCGGCA
CCTCAACTATTGTAATGTTTTCGGGCGCGGTTTGTGTTTGAACGGTCTGAGACGAGTGGCATTTTTTTCGTTTCTAATAGCT
TCCAAACAATTGTTGTCGTCGTTAAAGGTGCAGCGGTTGAGGTTCCGTCGGCATTTGGTGAGCGGGCGCAATTCAGACAT
CGATGGTGGTGGTGGGCGCTGGAATGTTAGGCACGGGAGAGGTTGGTGGCGGGTGCCCGGGTATATTTGTT
CTGTTTAGTTTGTTCGCGCACGATTGTGGCACCGCGCGAGCGCGCTGGCTGCACAAACGGAAGGTCGTCGCTTCGAGGC
AGCGCTTGGGTGGTGGCAATTCAATATTAATAATTGGAATACAAATCGTAAATAATCTGCTATAAGCATTTGTAATTTGCTATC
GTTTACCGTGGCGATATTAAACAACCGCTCAATGTAAGCAATTGTAATTGTAAGAGATTGTCTCAAGCTCCGACCGCGATAA
CAAGCCTTTTCAATTTTACAGCATTTGTAGTGGCGAGACACTTCGCTGTCGTCGACTCGAGTTCTATAGTGTACCTAAAT
CGTATGTGATGATACATAAGGTTATGATTAATTGTAGCCGCTTCTAACGACAATAAGTCCATATGGTGCACTCTCAGTAC
AATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCGACACCCGCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCC
CGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTGAGAGGTTTTCACCGTCAACCGAAACCGCGG
AGAGGAAAGGGCCTCGTGATACGCCCTATTTTATAGGTTAATGTGATGATAATAATGTTTCTTAGACGTGAGTGGCACTTT
TCGGGGAAATGTGCGGGGAACCCCTATTTGTTTATTTTCTAAATAACATTCAATATGATATCGGCTCATGAGACAATAACCTT
GATAAATGCTTCAATAATTTGAAAAAGGAAGAGTATGAGTATTCACATTTCCGTTGCGCCCTTATTCCTTTTGTGCGGCA

Figure 6D (Cont.)

TTTTGCCCTTCCTGTTTTTGTCTACCCAGAAAACGCTGGTGAAGATAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTA
CATCGAACTGGATCTCAACAGCGGTAAGATCTTTGAGAGTTTTTCGCCCCGAAGAACGTTTTTCCAATGATGAGCACTTTTAAAG
TTCTGCTATGTGGCGGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTGCGCCGATACACTATTCTCAGAAATGAC
TTGGTTGAGTACTCACAGTCA CAGAAAGCACTTTACGGATGGCATGACAGTAAGAGAAATTATGCAAGTGTGCCATAACCAT
GAGTGATAACACTGCGGCCAACTTACTTCTGACAAACGATCGGAGGACCGAAGAGCTAACCCGTTTTTTTGCACAAACATGGGGG
ATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAAACGACGAGCGTGACACACGATGCCCTGTGA
GCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGCAACAATTAATAGACTGGATGGA
GGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGTGGTTTTATTGCTGATAAATCTGGAGCCGGTGAGC
GTGGGTCTCGCGGTATCATTCAGCACTGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAG
GCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCCTCACTGATTAAAGCATTTGTTAACTGTCAAGCTTTA
CTCATATATACTTTAGATTGATTTAAAACTTCAATTTTAAAGGATCTAGTGAAGATCCTTTTGTGATAATCTCATGA
CCAAAATCCCTTAACTGAGTTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTT
TTTCTGCGCGTAATCTGCTGTGCTTGCAAAACAAAAAACCAACCGCTACAGCGGTGGTTTTGTTGCCGGATCAAGAGCTACCAAC
TCTTTTTCGGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAATACTGTCTTCTAGTGTAGCCGTAGTTAGGCCACCACT
TCAAGAACTCTGTAGCACCCGCTACATACCTCGCTCTGCTAATCTGTTAACAGTGGCTGTGCCAGTGGCGATAAGTCTGT
CTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGCGCAGCGGTGCGGCTGAACGGGGGGTTCGTGCACACAGCCAG
CTTGGAGCGGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGG
CGGACAGGTATCCGGTAAGCGGCAAGGTCCGAA CAGGAGAGCGCACGAGGAGCTTCCAGGGGGAAAACGCCCTGGTATCTTTAT
AGTCCTGTGCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGC
CAGCAACGCGGCCCTTTTACGGTTCTGCGCCCTTTTGTGCGCTTTTGTCTCACATGTTCTTTCTGCGTTATCCCTGATTTCTG
TGGATAACCGGTATTACCGCTTTGAGTGAGCTGTATACCGCTCGCCGAGCCGAAACGACCGGCGCAGCGAGTCAGTGAGCGAG
GAAGCGGAAGAGCGCCCAATA CGCAACCGCCCTCTCCCGCGCGTTGGCCGATTCAATTAATGCAGGTTAACCTGGCTTATCGA
AATTAATACGACTCACTATAGGAGACCGGCAGATCGATCTGTCTGA